PREVALENCE, PHENOTYPING AND MOLECULAR DETECTION OF *bla*NDM-1 and *bla*OXA-51 GENES IN CARBAPENEMASE PRODUCING STRAINS AMONG THE CARBAPENEM RESISTANT ENTEROBACTERIACEAE

Dissertation submitted in partial fulfillment of the

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CERTIFICATE

This is to certify that the dissertation entitled, **"PREVALENCE, PHENOTYPING AND MOLECULAR DETECTION OF *bla*NDM-1 and *bla*OXA-51 GENES IN CARBAPENEMASE PRODUCING STRAINS AMONG THE CARBAPENEM RESISTANT ENTEROBACTERIACEAE"** by **DR.M.JANE ESTHER**, Post graduate in Microbiology (2014-2017), is a bonafide research work carried out under our direct supervision and guidance and is submitted to The Tamilnadu Dr. M.G.R. Medical University, Chennai, for M.D. Degree Examination in Microbiology, Branch IV, to be held in March 2017.

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DECLARATION

I hereby declare that this dissertation entitled **"PREVALENCE, PHENOTYPING AND MOLECULAR DETECTION OF *bla*NDM-1 AND *bla*OXA-51 GENES IN CARBAPENEMASE PRODUCING STRAINS AMONG THE CARBAPENEM RESISTANT ENTEROBACTERIACEAE"**, is a bonafide and genuine research work carried out by me, **DR.M.JANE ESTHER**, under the guidance of **Dr.A.Uma**, Professor and Head, Department of Microbiology, Chennai Medical college hospital and research centre

The dissertation is submitted to The Tamil Nadu Dr.M.G.R Medical University towards the partial fulfillment of requirements for the award of M.D Degree (Branch IV) in Microbiology.

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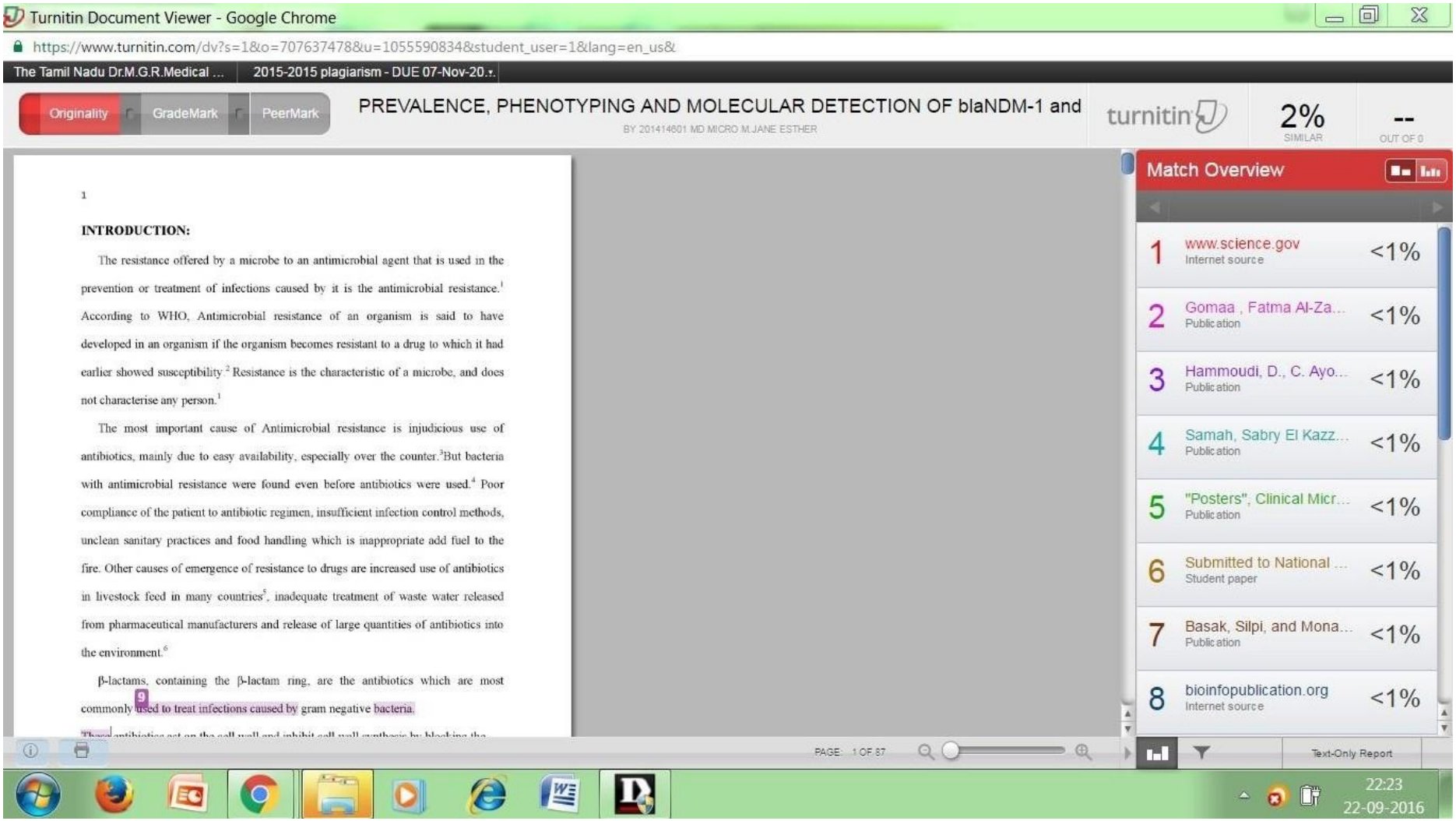
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INTRODUCTION:
The resistance offered by a microbe to an antimicrobial agent that is used in the prevention or treatment of infections caused by it is the antimicrobial resistance.[1] According to WHO, Antimicrobial resistance of an organism is said to have developed in an organism if the organism becomes resistant to a drug to which it had earlier showed susceptibility.[2] Resistance is the characteristic of a microbe, and does not characterise any person.[1]
The most important cause of Antimicrobial resistance is injudicious use of antibiotics, mainly due to easy availability, especially over the counter.[3]But bacteria with antimicrobial resistance were found even before antibiotics were used.[4] Poor compliance of the patient to antibiotic regimen, insufficient infection control methods, unclean sanitary practices and food handling which is inappropriate add fuel to the fire. Other causes of emergence of resistance to drugs are increased use of antibiotics in livestock feed in many countries[5], inadequate treatment of waste water released from pharmaceutical manufacturers and release of large quantities of antibiotics into the environment.[6]
β-lactams, containing the β-lactam ring, are the antibiotics which are most commonly used to treat infections caused by gram negative bacteria.
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ABBREVIATIONS

AMR	Antimicrobial resistance
WHO	World Health Organisation
CRE	Carbapenem Resistant Enterobacteriaceae
MDR	Multi Drug Resistant
E.coli	*Escherichia coli*
K.pneumoniae	*Klebsiella pneumoniae*
K.oxytoca	*Klebsiella oxytoca*
K.rhinoscleromatis	*Klebsiella rhinoscleromatis*
K.ozaenae	*Klebsiella. ozaenae*
E.aerogenes	*Enterobacter aerogenes*
Citrobacter spp.,	*Citrobacter species*
S.marcescens	*Serratia marscescens*
Proteus spp.,	*Proteus species*
S.Typhi	Salmonella Typhi
DNA	Deoxyribo Nucleic Acid
RNA	Ribonucleic acid
HAI	Hospital Acquired Infection
BAP	Blood agar plate
MAP	Mac Conkey agar plate
CA	Chocolate agar
MR	Methyl Red
VP	Voges Proskaeur

TSI	Triple sugar iron
KPC	*Klebsiella pneumoniae* Carbapenemase
MHT	Modified Hodge Test
CDT	Combined disc test
EDS	EDTA disk synergy test
ESBL	Extended spectrum beta lactamse
MBL	Metallo beta lactamase
IMP	Imipenamase metallo betalactamase
VIM	Verona integron mediated metallo betalactamase
UTI	Urinary tract infections
SSI	Surgical site infections
CRBSI	Catheter Related Blood Stream Infections

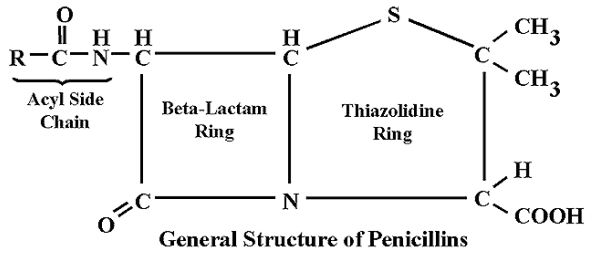
1. INTRODUCTION

The resistance offered by a microbe to an antimicrobial agent that is used in the prevention or treatment of infections caused by it is called antimicrobial resistance.[1] According to WHO, Antimicrobial resistance of an organism is said to have developed in an organism, if the organism becomes resistant to a drug to which it had earlier showed susceptibility.[2] Resistance is the characteristic of a microbe, and does not characterise any person.[1]

The most important cause of Antimicrobial resistance is injudicious use of antibiotics, mainly due to easy availability, especially over the counter.[3]But bacteria with antimicrobial resistance were found even before antibiotics were used.[4] Poor compliance of the patient to antibiotic regimen, insufficient infection control methods, unclean sanitary practices and food handling which is inappropriate add fuel to the fire. Other causes of emergence of resistance to drugs are increased use of antibiotics in livestock feed in many countries[5], inadequate treatment of waste water released from pharmaceutical manufacturers and release of large quantities of antibiotics into the environment.[6]

β-lactams, containing the β-lactam ring, are the antibiotics which are most commonly used to treat infections caused by gram negative bacteria.

Figure 1 Structure of β-lactam antibiotic



These antibiotics act on the cell wall and inhibit cell wall synthesis by blocking the transpeptidase enzyme.[7]

Among the β-lactam antibiotics, there are various classes depending upon the nature of the heteroatom included in the cycle – Penicillins, Cephalosporins, Monobactams and Carbapenems.[7] Of these, the Carbapenems are the drugs of last resort which are used in treating Multidrug resistant infections unresponsive to treatment with the other classes of β-lactam antibiotics.[8]

Enterobacteriaceae are gram negative human pathogens causing various community acquired and hospital acquired infections like fever, blood stream infections, urinary tract infections, infections of lung, abdomen, CNS and device-associated infections.[8]They can easily spread between humans through contaminated hand, water and food. Transposons and plasmids mediate transfer of genes horizontally.[8]

Community-acquired *Enterobacteriaceae* isolates producing Extended-spectrum betalactamases are spreading and have been reported worldwide.[8] β-lactams with inhibitor combinations are being increasingly used in the treatment of such bacterial infections.[9]But organisms resistant even to these combinations have dramatically increased. Carbapenems were used to treat such organisms. Recently, organisms resistant even to Carbapenems are on the rise.

In the recent past, Carbapenem-resistant *Enterobacteriaceae* are being identified and brought to light in all countries across the world principally because of acquirement of genes that are responsible for Carbapenemase production.

The Carbapenemases are betalactamases that are capable of inactivating or hydrolysing the Carbapenem group of betalactam antibiotics. This is the main cause of Carbapenem resistance in gram negative bacilli.[10] Hyperproduction of enzymes called Amp C betalactamases can also result in resistance to Carbapenems.[10]

The Carbapenemases, by hydrolysing or inactivating Carbapenems have deprived us of the last choice of antibiotics, the Carbapenems, there by posing a serious threat to the patient and the physician by increasing morbidity and mortality due to infections with Multi-drug resistant (MDR) organisms[8].

β-lactamases are classified in molecular and functional ways as described by Ambler's and Bush Jacoby classifications respectively[11]. Of these, the Metalloβ-lactamases and Carbapenemases are the newly discovered β-lactamases.[12]The Carbapenemases have been broadly classified into Serine Carbapenemases and Metalloβ-lactamases based on the reactive sites of the enzymes.[10]

The classes of Carbapenemases with examples have been depicted in the table below.

Table 1 Classification of Carbapenemases[10]

Class of Carbapenemase	Active site ingredient	Examples
Class A	Serine	KPC, NMC,GES,IMI, SME
Class B	Metal ion(Zinc)	IMP, NDM, VIM
Class D	Serine	OXA 48, OXA 53

Carbapenemases have been increasingly reported in bacteria of the *Enterobacteriaceae* family[8]. These drug resistant organisms pose a real challenge to

the patient and the treating physician. The former has to face the toxic effects of the drugs if higher doses are needed. The later has to face a challenge in choosing an alternative drug to treat the patient and to get a successful outcome.

Finding out patients and the carriers colonised/infected with *Enterobacteriaceace* that produce Carbapenemases can help to a great extent in preventing further spread of these resistant organisms. A special emphasis has been given to *Enterobacteriaceae* because they are the most common organisms causing community acquired and nosocomial infections.[8]

Carbapenemases produced by the resistant bacteria can be detected by various phenotypic tests and confirmed by genotyping.

Ours is a tertiary care seven hundred and seventy bedded hospital attached to medical college catering mostly to rural population with an out-patient turnover of around thousand three hundred patients per day.

Our aim was to detect the prevalence of Carbapenemase producing strains among the Carbapenem resistant *Enterobacteriaceae*. This type of study has not been done in this area, which is predominantly a rural population and that is the reason why this study was undertaken.

2. AIMS AND OBJECTIVES

- To detect the prevalence of Carbapenemase producers among the Carbapenem resistant *Enterobacteriaceae* using various phenotypic methods.
- Molecular detection of *bla*NDM-1 and *bla*OXA-51 genes among the Carbapenemase producers.
- To compare various phenotypic and genotypic methods and to determine the efficacy of each phenotypic test.

3. REVIEW OF LITERATURE

3.1 HISTORICAL PERSPECTIVES OF ENTEROBACTERIACEAE:

Enterobacteriaceae are gram negative bacilli that inhabit the intestines of humans and animals and are called “Enteric bacteria” or “Enterobacteria”. Otto Rahn first proposed the name *Enterobacteriaceae* in 1937 for a group of organisms which were similar in their morphological and biochemical aspects, within a single genus called *Enterobacter*.[13]

Borman, Stuart and Wheeler (1944) defined the family as gram negative bacilli that were non sporing and widely distributed in nature, fermenting glucose forming acid alone or acid with gas, reduce nitrates to nitrites. When motile the flagella are peritrichous.[14]

The scientific classification of *Enterobacteriaceae* is as follows.

Table 2 Scientific classification of *Enterobacteriaceae*[13]

Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	*Enterobacteriaceae*

Members of *Enterobacteriaceae* reside in the intestine of humans and animals and other mammalian hosts as symbionts and commensals. They have the capacity to synthesize vitamin K, which cannot be synthesised by their hosts by themselves. However, some of them may become pathogenic, causing diseases like gastroenteritis and urinary tract infections[14].

3.2 DISCOVERY OF THE MEMBERS OF *ENTEROBACTERIACEAE*:

Serratia was the first member of *Enterobacteriaceae* to be discovered in 1819 by Bizio on an Italian dish. Subsequently other members were discovered.[14]

Table 3 Discovery of the members of Enterobacteriaceae[14]

Member (Genus)	Year of discovery	Name of the discoverer
Serratia	1819	Bartolomeo
Providencia	1852	Robert Bowley
Klebsiella	1882	Carl Friedlander
E.coli	1885	Theodore Escherich
Salmonella	1885	Daniel E Salmon
Proteus	1885	Gustav Hauser
Yersinia pestis	1894	Alexandre Yersin
Shigella	1896	Kiyoshi Shiga
Morganella	1906	Morgan
Citrobacter	1932	Werkman and Gillen
Enterobacter	1960	Hormache & Edwards
Edwardsiella	1962	Frank

3.3 CLASSIFICATION OF *ENTEROBACTERIACEAE:*

- Members of *Enterobacteriaceae* were first classified in 1893 by Theobald Smith into Lactose fermenters and lactose nonfermenters. Ability to ferment lactose distinguishes enteric from obligate aerobes and is of practical value in diagnostic microbiology.[14] Lactose fermenters include *Escherichia,*

Enterobacter, *Citrobacter* and *Klebsiella*. Non lactose fermenters are *Salmonella , Shigella, Yersinia and Proteus.*[14]

- Cowan in 1956 classified *Enterobacteriaceae* into six genera: *Salmonella, Escherichia, Shigella, Citrobacter, Klebsiella and Proteus*[15]
- Ewing (1960,1966) classified *Enterobacteriaceae* into four tribes: *Escherichiae, Klebsiellae, Proteeae and Erwiniae.*[16]
- The three widely used systems for the classification of *Enterobacteriaceae* are Bergey's, Kauffmann and Edwards-Ewing. They have certain differences, but their general approach is the same.[16]

Table 4 Edwards-Ewing classification of *Enterobacteriaceae*[16]

Tribe	**I.Escherichiae**	**II.Klebsielleae**	**III.Proteeae**	**IV.Erwinieae**
Genus	*1.Escherichia* *2.Edwardsiella* *3.Citrobacter* *4. Salmonella* *5. Shigella*	*1.Klebsiella* *2.Enterobacter* *3.Hafnia* *4.Serratia*	*1.Proteus* *2.Morganella* *3.Providencia*	*1.Erwinia*

- Between 1950s and 1970s, methods like chemotaxonomy, Carbon utilization assay and phage typing were used for classification and identification.[18]
- DNA hybridization was pioneered by Don Brenner at CDC in early 1970s as the gold standard for determining relatedness among bacteria. [18]
- Currently 16sRNA sequencing is being widely used to identify and classify *Enterobacteriaceae* and bacteria which could not be done by conventional methods.[18]

3.4 PROPOSED CHANGES IN *ENTEROBACTERIACEAE*:

- Based on 16 S rRNA studies, *Plesiomonas* was found to be more related to *Enterobacteriaceae* than to *Vibrionaceae* family and so it has been classified into *Enterobacteriaceae* family.[18]
- Three species of *Klebsiella* that has been assigned to *Raoultella* are *K.ornitholytica, K.terrigena, K.planticola.*[19]
- *Calymmatobacterium granulomatis* has been assigned to *Klebsiella* family[19]
- *Enterobacter sakazakii* was identified as a new species in 1980. [19]

3.5 *ENTEROBACTERIACEAE* AND INFECTION:

Enterobacteriaceae is the most medically important bacterial family which includes bacteria capable of causing infections in large numbers in community as well as in hospitals. They are associated with infections involving the gastrointestinal, urinary tract, skin and soft tissues, wound, meninges and blood stream[20].

3.5.1 CLINICAL MANIFESTATIONS OF *ENTEROBACTERIACEAE* INFECTIONS:

The coliform bacilli (genera *Escherichia, Enterobacter, Klebsiella, Serratia, Proteus* and *Citrobacter)* are potential pathogens that can cause a number of infections. *Escherichia coli* is the member of *Enterobacteriaceae* that is most commonly isolated in the clinical laboratory.[21]The infections caused by the bacteria belonging to *Enterobacteriaceae* are as follows.

- **Enteric Infections***:* In developing countries, the most common enteric pathogen is *E.coli.* The enteric pathogens include, classical enteropathogenic, enterotoxigenic, enterohemorrhagic, enteroinvasive and enteroggregative strains.[21]

- **Nosocomial Infections*:*** Around 30 percent of HAIs in United States is due to *Coliform* and *Proteus* bacilli. The major sites of hospital acquired infections are the urinary tract, bloodstream, lung (pneumonias) and surgical sites. These nosocomial pathogens are responsible for more than 40% of the urinary tract and more than 20% of surgical site infections. They also contribute to more than 15% of the blood stream infections and about 30% of the lung infections. *E coli* is the most common pathogenic cause of hospital acquired infections.[21]

- **Community-Acquired Infections***:* Most cases of UTI are caused by *Escherichia coli.* Other members of *Enterobacteriaceae* associated with UTI are *Klebsiella, Proteus* and *Enterobacter* species. *Proteus mirabilis* is the most common cause of complicated urinary tract infections. *K.pneumoniae*, *K.ozaenae and K.rhinoscleromatis* cause pneumonia, ozena and rhinoscleroma respectively.[21]

3.6 TREATMENT OPTIONS FOR *ENTEROBACTERIACEAE* INFECTIONS:

- Common antibiotics used for Gram negative bacterial infections are Betalactams, Aminoglycosides, Macrolides and Fluoroquinolones.[22]

Table 5 Treatment options for *Enterobacteriaceae* infections

Organism	Clinical condition and drugs used in treatment
E.coli	Cystitis – Fluoroquinolones or nitrofurantoin Meningitis- Ceftriaxone Pneumonia- 3rd gen Cephalosporins or Fluoroquinolones Cholecystitis – Third gen cephalosporins Intra abdominal abscess- piperacillin-tazobactam, imipenem, cilastatin[23]
Klebsiella	UTI – oral quinolones, third gen cephalosporins Community acquired pneumonia – third gen cephalosporins and quinolones Nosocomial pneumonia – Aminoglycosides in addition to the above mentioned drugs[24]
Proteus	Uncomplicated UTI – Quinolones or Cotrimoxazole Complicated UTI – Ceftriaxone, Gentamycin, Cotrimoxazole, Aztreonam[25]
Enterobacter	Betalactams, Carbapenems, Fluoroquinolones, Aminoglycosides [26]

3.7 BETALACTAM ANTIBIOTICS:

3.7.1 STRUCTURE AND ACTION OF BETALACTAMS:

- Betalactam antibiotics are a class of antibiotics containing the betalactam ring. The betalactam ring is a four membered cyclic amide consisting of three carbon (3 C) atoms and one nitrogen (1 N) atom. It is named so because of the attachment of Nitrogen atom to the β-carbon in relation to the Carbonyl (C=O) group.[7]
- Betalactam antibiotics include Penicillins, Cephalosporins, Monobactams and Carbapenems[7].
- Betalactam antibiotics are cell wall precursor analogues, which act by binding with PBPs (Penicillin binding Proteins) in bacterial cell wall and inhibiting new cell wall peptidoglycan synthesis mediated by the transpeptidase enzyme.[7] Furthermore, binding of betalactam drugs to PBP triggers the cell wall hydrolases, which cause lysis of bacterial cell wall[7].

3.8 EVOLUTION OF DRUG RESISTANCE IN *ENTEROBACTERIACEAE*:

The ability of the bacteria to survive and multiply withstanding the effect of antibiotic is termed as antibiotic resistance.[27] *E.coli* is the organism from which the first betalactamase was detected, even before penicillin came into use in medical practice[27].

Table 6 Evolution of drug resistance in *Enterobacteriaceae*

Year	Event (Antimicrobial resistance)
1937	Sulfonamides introduced for treatment[28]
1940	Penicillin came into clinical use[29]
1940	First evidence of betalactamases (Penicillinase) demonstrated in *E.coli* by Abraham and Chain[29]
1940	Tetracycline came into clinical use[30]
1953	First tetracycline resistance was reported in *Shigella dysentriae*[30]
1970s	Plasmid mediated β-lactamases assumed importance in *Enterobacteriaceae* and other gram negative bacteria[31]
1972	First epidemic of Chloramphenicol resistant *Salmonella* in Kerala reported by Paniker et al.[32]
1989	MDR *S.Typhi* outbreaks resistant to Chloramphenicol, Ampicillin, Trimethoprim, Streptomycin, Tetracycline and Sulfonamides were reported in India and Pakistan[32]
1992	*S.Typhi* resistant to Ciprofloxacin was first reported in UK.[32]
1970-80s	Development of broad spectrum Cephalosporins, Cephamycins, Monobactams and Carbapenems[29]
1990	Inducible chromosomally mediated β-lactamases among gram negative bacteria[29]

E.coli resistance to the antibiotic of last resort – colistin has now been reported in US (2016)[33].The increasing resistance among these organisms are indicative of changes in the frequency of antibiotic resistance determining genes.[34]

3.9 CARBAPENEM RESISTANT *ENTEROBACTERIACEAE (CRE)*:

Enterobacteriaceae resistant to treatment with Carbapenems are called Carbapenem resistant *Enterobacteriaceae*. They are difficult to treat organisms because of their high resistance, even to the last resort antibiotics – The Carbapenems.[35] *Klebsiella* and *E.coli* are the most commonly encountered *CRE*.[35] The two mechanisms of resistance in *CRE* are Carbapenemases (Betalactamases that hydrolyse Carbapenems) and Cephalosporinases along with porin loss.[10]*Klebsiella pneumoniae* Carbapenemase (KPC), NDM (New Delhi Metallo-betalactamase) and VIM (Verona Integron-Mediated Metallo-β-lactamase) are some of the Carbapenemases.[10]

3.10 TYPES OF DRUG RESISTANCE:

- Drug resistance can be genetic or non genetic. The genetic drug resistance can be chromosomal or extra chromosomal according to the location of resistance determining gene. [27]
- Mutations in such genes coding antibiotic target site proteins cause chromosomal resistance.[27] Thus alteration in the penicillin binding proteins (PBPs) result in resistance to betalactam antibiotics. These resistant genes are transferred to the bacterial progeny during replication, but not transferable from one species to the other. [27]
- Plasmids and transposons are extra chromosomal mobile genetic elements which can carry resistant genes for antibiotics, for example plasmid genes encoding β-lactamases. In contrast to chromosomal genes, extra chromosomal genes are transferable by conjugation, transformation and transduction.[27]

3.11 MECHANISMS OF ANTIBIOTIC RESISTANCE IN GRAM NEGATIVE BACILLI:

Antimicrobial resistance by a microbe can occur through any of the following mechanisms.[27]

- Inactivation of the antibiotic by enzyme production (Example**.** Betalactamase enzyme production) - the commonest mechanism of resistance.
- Target site alteration such that the binding capacity of the antibiotic is reduced. (Example. Production of new Penicillin Binding Proteins)
- Modification of metabolic pathways,
- Active efflux pumps that throw away the antibiotic out of the cell resulting in reduced intracellular concentration of the drug.[27]

3.12 BETALACTAMASES:

Enzymes which inactivate betalactam antibiotics by hydrolyzing the nitrogen-carbonyl bond in their betalactam ring are collectively known as betalactamases. They can be plasmid mediated or chromosomal.[7]These β-lactamases are secreted as exozymes in gram positive bacteria and within the periplasmic space in bacteria that are gram negative.[7]

3.12.1 CLASSIFICATION OF BETALACTAMASES:

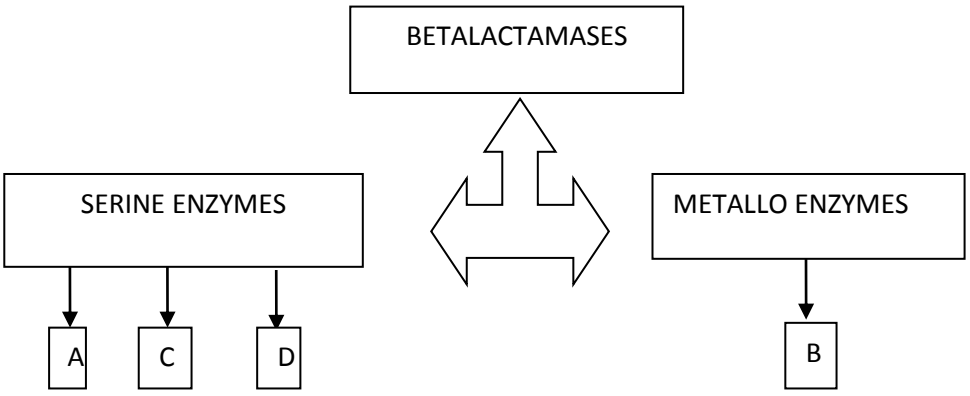
There are two popular classifications for betalactamases - Bush Jacoby Medeiros functional classification and Ambler's molecular classification.[7]

3.12.1.1 AMBLER'S MOLECULAR CLASSIFICATION

This system categorizes all betalactamases into four classes (A-D) based on protein homology (nucleotide and amino acid sequence) instead of phenotypic characters. Enzymes of Classes A, D and C have serine at their active site and are called serine betalactamases. In contrast, class B enzymes require zinc for their activity and hence known as Metallo-betalactamases. ESBL enzymes belong to Ambler class A and D[12].

- **Class A**: Large proteins (MW 29,000), common in GNB, preferentially hydrolyse penicillins, e.g. TEM-1[12]
- **Class B:** Metalloenzymes (require zinc), common in *P. aeruginosa*, *Acinetobacter spp.*, not inhibited by clavulanic acid but chelation with zinc renders it inactive. [12]
- **Class C:** Cephalosporinase activity, has structural homology with PBPs, large proteins (MW 39,000), e.g. AmpC of *E. Coli.*[12]
- **Class D:** Hydrolyse oxacillin, plasmid mediated e.g. OXA-1.[12]

Figure 2 Ambler's molecular classification of β-lactamases[12]



Ambler's molecular classification of Betalactamases

3.12.1.2 BUSH-JACOBY-MEDEIROS FUNCTIONAL CLASSIFICATION[11]

Table 7 Bush-Jacoby-Medeiros classification scheme for betalactamases with corresponding Ambler classes

Bush-Jacoby Group	Sub groups	Enzyme type	Molecular Class	No. of enzymes	Clavulanate inhibition	Examples
1	---	Cephalosporinase †*	C	53	no	*E.cloacae*
2	2a	Penicillinase*‡	A	20	yes	*S.aureus, S.albus*
	2b	Broad Spectrum†*	A	16	yes	TEM-1,SHV-1
	2be	Extended Spectrum*	A	38	yes	TEM-3,SHV-2
	2br	Inhibitor Resistant	A	9	Diminished	TEM-30,TRC-1
	2c	Carbenicillinase	A	15	Yes	PSE-1,CARB-3,BRO-1
	2d	Cloxacilliase	Dor A	18	Yes	
	2e	Cephalosporinase	A	19	Yes	*Proteus*
	2f	Carbapenemase §	A	3	Yes	*E.clocae* IMI-1. NMC-A
3	---	Metalloenzyme ɣ	B	15	No	*S.maltophila* L1
4	---	Penicillinase		7	No	*P. cepacia*

Location of betalactamase gene on † bacterial chromosome; *plasmid; ‡ transposon

§ Serine Carbapenemase; ɣ Zinc based Carbapenemase also known as metallo betalactamase

It was introduced by Bush Jacoby and Medeiros in 1995. It is based on substrate and inhibitor profile. Since it takes into consideration the interaction between betalactam drugs and betalactamase inhibitors with these enzymes, it is more relevant to a

physician or microbiologist[11].

The current system includes classes C, A and D betalactamases which belong to functional group 1,2 and 3 respectively.[11]

3.13 CARBAPENEMASES:

Carbapenemases are betalactamases that cause resistance to the Carbapenems, the β-lactam group with the broadest spectrum of antibacterial action. Carbapenems were less susceptible to the inactivating activity of many betalactamases till the recent past. But now even these efficient antibiotics are becoming susceptible to the enzymatic inactivation by betalactamases.[10]

The enzymes hydrolysing Carbapenems can be grouped into classes A or B by molecular analysis. The former has serine as the active site member and the latter has zinc at the active site.[10] since these enzymes are dependent on zinc, a metal, they are called Metallobetalactamases.[10]Some class C Cephalosporinases can hydrolyse/ inactivate Carbapenems and result in Carbapenem resistane, but they are not called Carbapenemases because they are not Carbapenem specific.[10]

3.14 NATURAL OCCURRENCE OF CARBAPENEMASES:

In 1993, the first Carbapenemase producing *Enterobacteriaceae* (NmcA) identified was *Enterobacter*,[10] following which, a number of types of Carbapenemases have been identified. Carbapenemase producers in general produce more than one type of betalactamase, making them resistant to many other groups of antibiotics in addition to Carbapenems.[10] Two class A enzymes in *Enterobacter cloacae* isolates and

four class A enzymes from *Serratia marscescens* isolates were identified.[10] Serine Carbapenemase producers were found to produce class C Cephalosporinases in addition to that. Therefore group 2f enzymes were called as "secondary β-lactamases," by Livermore.[10]

Metalloenzymes that were mediated through plasmids were identified from Japan, after the description of chromosome mediated enzymes, before the last decade of the twentieth century.[10]All gram negative bacteria, including the *Enterobacteriaceae* and *Non fermenters* were found to produce these enzymes.

Carbapenem mediated increased selection pressure can result in the spread of these plasmid mediated Metallobetalactamases much faster than one could predict.[10]

3.15EPIDEMIOLOGY OF CARBAPENEMASES:

International travel increases the chance of infection/colonisation with antibiotic-resistant organisms with a higher risk for people travelling to India, the Middle East and Africa[10]. *Klebsiella pneumoniae* Carbapenemases (KPC) were initially reported in United States in various members of *Enterobacteriaceae*, following which they were reported in other countries like India, China and all over the world at later stages.[10]

Bacterial isolates producing Guiana extended spectrum (GES) Carbapenemases have been reported all over the world, after having been initially reported in European and Asian countries. However, this enzyme is comparatively rare.[10]

Verona integron mediated (VIM-1) Carbapenemases and Imipenem hydrolysing betalactamases (IMI-1) have been reported mainly from Asian,

African and European countries and also across the world. Sao Paulo Metalloenzymes (SPM) were detected in Brazil initially and they still remain confined to Brazil. Seoul imipenemase and German imipenemase enzymes are also confined to the places where they originated.[10]

Verona integron mediated enzymes were the most frequently isolated Metalloenzymes till New Delhi Metallobetalactamases (NDM) took hold of that place in 2008, when the first NDM producing isolate was detected in a patient in Sweden who was originally from India. [10] From 2009, there has been a substantial increase in the incidence and detection of New Delhi Metallobetalactamases from all over the world. Since *bla*NDM-1 gene is located on a genetic element which is highly mobile, it can spread easily, which has resulted in rapid spread of the same gene from Asian countries like India to the United States and all over the world, probably due to medical tourism. Hospitalisation in India has been proposed to be a major determinant in acquiring NDM genes.[10]

Oxacillinases capable of hydrolysing Carbapenems are mainly confined to *Non fermenters* like *Acinetobacter baumanii*. But there are reports of oxacillinases being isolated from *Enterobacteriaceae* isolates like *Klebsiella pneumoniae* from Asian and American countries. Some of the oxacillinases are OXA-58, OXA-48, OXA-51 and OXA-23.[10]

Thus drug resistant *Enterobacteriaceae* are widely spread throughout the world, with NDM being predominant in India.[10]

3.16 PRODUCTION OF CARBAPENEMASES:

There can be numerous patterns for the production of several betalactam hydrolysing enzymes. MBL gene expression may be inducible or may not be inducible.

- Class A Carbapenemases have an inducible expression. Cephalosporinases that are induced by imipenem is a good example for the above. Amp C betalactamases are induced by Cefoxitin.[36]
- However, it is not known if the actual inducer is the betalactam antibiotic itself or the destruction of cell wall as a result of the action of betalactam antibiotic on PBPs.[36]
- Alterations affecting the promoter region of the gene and also in the insertion sequence (IS) element lead to drug resistance in an organism.[36] The insertion sequence element causes increased transcript expression because of the signals it can generate and initiate transcription.[37]
- If the genes that encode for Carbapenemases are encoded/ located on chromosomes, they spread very slowly but if they are located on plasmids, they spread rapidly as in case of plasmid encoded Metallobetalactamase gene in *Klebsiella*.[36]

3.17 MOST COMMON TYPES OF CARBAPENEMASES

Table 8 Most common types of Carbapenemases[10]

Class	Carbapenemase	*Enterobacteriaceae*	*Nonfermenters*
A1	KPC	+++	+
B(Metallobetalactamases)	NDM, VIM, IMP	+++	+++
D	OXA-48	+++	+/-

3.17.1 CHROMOSOMAL AND PLASMID ENCODED ENZYMES AMONG THE DIFFERENT CLASSES:

A. Molecular ClassA enzymes:

Chromosomally encoded enzymes:

SME (for *Serratia marscescens*), IMI (for imipenem hydrolyzing β-lactamase) and NMC (for Non Metalloenzyme Carbapenemase) are chromosomally encoded. [10]

Plasmid encoded Carbapenemases:

KPC and GES are plasmid encoded classA Carbapenemases.[10]

KPC (for *Klebsiella pneumoniae* Carbapenemase):

Though KPC are predominant in *K.pnemoniae*, these betalactamases were also found to be the cause of drug resistance in other members of *Enterobacteriaceae* like *Salmonella* and *E.coli*. The organisms producing these Carbapenemases are resistant to all Monobactams and Carbapenems in addition to penicillins and Cephalosporins, but they are susceptible to betalactamase inhibiting drugs like Piperacillin-Tazobactam. The genes responsible for the production of *Klebsiella pneumoniae* Carbapenemases are horizontally transferred, confering KPC enzyme with an extraordinary spreading capacity.[10]

B. Molecular Class B enzymes:

Chromosome borne MBLs:

BCI, BCII from *Bacillus cereus*, CphA from *Aeromonas spp*, L1 from *Stenotrophomonas maltophilia*, *Bacteroides fragilis* (CcrA) are chromosome mediated.[10]

Plasmid borne MBLs:

Metallobetalactamases that are plasmid borne have gained importance because of their tendency to spread all over the world. Verona integron mediated Metallobetalactase, Imipenem hydrolysing betalactamase, Guiana extended spectrum betalactamase, Seoul imipenemase and NewDelhi Metallobetalactamase are some of the plasmid mediated Metallobetalactamases. Of these, NDM is the most common one in India.[10]

New Delhi Metallobetalactamase was reported for the first time in a patient in Sweden who was an Indian by origin and had also been hospitalised in India for treatment.[10] *Klebsiella pneumoniae* was the bacteria isolated from him.[10] NDM-1 enzyme is now found to spread worldwide in a rapid manner. Outbreaks due to NDM were mostly linked to India and Balkan countries. NDM-1, NDM-2 to NDM-6 and NDM-7 are the variants of NewDelhi Metallobetalactamases. These enzymes spread rapidly between bacteria, because of their location on plasmids. The strains harbouring NDM show a broad spectrum of resistance to other betalactam antibiotics, which has left us with very meagre treatment options. Their prevalence in *Enterobacteriaceae* all over the world is highly suggestive of their intense ability to spread.[10] Infection with Metalloenzyme producers have a high mortality rate exceeding sixty percent.[10]

C. Class D enzymes:

Oxacillinases are enzymes which inactivate Oxacillin. OXA-23 and OXA-48 are plasmid borne and OXA-50 and OXA-54 are chromosome encoded.[10]

3.18 LABORATORY DETECTION OF CARBAPENEMASES:

CLSI suggested detecting Carbapenemases for epidemiological and academic purposes rather than routine testing.[37]

3.18.1. SCREENING TESTS FOR CARBAPENEMASES (PHENOTYPIC METHODS):

3.18.1.1 SCREEN AGARS FOR CARBAPENEMASE PRODUCERS:

Chromogenic media like KPC CHROM agar, Brilliance CRE and Hardy CHROM Carbapenemase agar are commercially available to screen for Carbapenemase production in gram negative bacilli.

KPC CHROME AGAR:

Rapid detection of CRE is very much needed, for patients who are hospitalised in *CRE* endemic areas. Culturing bacteria on MacConkey agar which is supplemented with Carbapenem is a cheap and easy method for detection of *CRE*, but takes more time. The sensitivity and specificity of PCR is very high but the cost and other factors denies us of it being used routinely.[38]

Chromogenic media CHROM agar has been prepared and recently introduced with agents that selectively allow Carbapenem resistant isolates to grow. It has the capacity to inhibit all Carbapenem resistant and gram positive bacterial isolates.[38] The suspected Carbapenemase producers have to be inoculated in the media and incubated overnight as for other bacterial cultures. CRE cultures take on different colours depending on the nature of enzymes they produce [38]Growth on KPC CHROM agar has a high sensitivity and specificity of 100% and 98.4%, respectively for detecting Carbapenemase producing Enterobacteriaceae.[38]

3.18.1.2 ANTIMICROBIAL SUSCEPTIBILITY TESTING – KIRBY BAUER METHOD:

- Antimicrobial susceptibility testing using Carbapenem antibiotic (Imipenem or Meropenem) provides a presumptive evidence of Carbapenemase production.[43]
- However, Carbapenemase producing *Enterobacteriaceae* are also resistant to one or more of the subclass III Cephalosporins. (Cefotaxime, Ceftazidime, Ceftriaxone, Cefoperazone). So susceptibility to third generation Cephalosporins are also screened for.[37]
- According to CLSI 2016, if the zone of inhibition around Meropenem (10 µg) or Imipenem (10 µg) disc is less than 23mm or if the MIC of the bacterial strain is less than 2µg/ml, it can be taken as a presumptive Carbapenemase producer.[37]
- Ertapenem disc or Meropenem disc is recommended by CLSI for Carbapenemase screening as Imipenem is less effective in Carbapenemase detection when compared to the other two drugs.[37]
- Most of the serine Carbapenemases are sensitive to the third and fourth generation Cephalosporins, Monobactams and Carbapenems but not susceptible to betalactamase inhibitors, where as the Metalloenzymes are very much sensitive to drugs like Aztreonam and have a poor susceptibility to Cephalosporins of third and fourth generation and to Carbapenems.
- When Extended spectrum betalactamases, Ambler class C betalactamases are co-produced with Carbapenemases, the test is likely to become less specific, which makes it imperative to screen for the other betalactamases also.[37]

3.18.1.3 TEN DISC METHOD

This procedure helps in screening of an *Enterobacteriaceae* isolate for all β-lactamases (ESBLs, AmpC and Carbapenemases).[45]Aztreonam (30), Cefotaxime(30) , Ceftazidime (30), Cefotaxime + clavulante (30/10), Ceftazidime + clavulante (30/10), Ceftriaxone (30), Cefoxitin (30), Cefepime, Ertapenem (10) and Imipenem(10) are the drugs for which the sensitivity of the organism is detected, by using Kirby Bauer disc diffusion assay. [39]

- **Detection of ESBLs:**

  Ceftazidime or Cefotaxime discs with and without clavulanate are used to detect ESBLs. If the zone increases by 5mm or above with clavulanate combination, the isolate is an ESBL producer.[39]

- **Detection of AmpC β-lactamases:**

  Amp C β-lactamases are resistant to Cefoxitin and Cefotetan. High level AmpC producers are even resistant to Carbapenems and Aztreonam.[39]

- **Detection of Carbapenemases:**

  Ertapenem and Imipenem discs are used to screen for Carbapenemases. If an isolate is R to Ertapenem and S to Imipenem, is it a possible KPC producer.[39]

This ten disc procedure is a very useful screening test for all types of β-lactamases, which can then be confirmed by confirmatory tests.

3.18.1.4 MIC DETERMINATION:

MIC can be determined by using phenotypic methods like E-test. Other automated methods like Vitek, Microscan walkway are also used to determine MIC. However both these methods can produce results which are not consistent. There can be a few bacterial colonies within the zone of inhibition which makes the compatibility of E-test questionable in detection of MIC. Hyper production of Amp C or over expression of CTX-M can result in elevated MICs to Carbapenems.[10]

3.18.1.5 THE CARBA NP TEST:

Carba NP test is a test that differentiates Carbapenem resistance due to Carbapenemase production from that due to other mechanisms.[40]It is based on bacterial lysate mediated hydrolysis of Imipenem invitro, detectable by pH changes using the indicator phenol red from red colour to yellow or orange colour. The sensitivity and specificity of this test for detection of Carbapenemase production in *Enterobacteriaceae* is 100%.[40]

Isolated colonies of the suspected bacteria are mixed with a solution of Imipenem and phenol red indicator solution and incubated for about two hours at a temperature of thirty seven degrees. If Carbapenemase producers are present the Carbapenemase will hydrolyse the Imipenem, resulting in lowering of the pH which causes a change in colour of the phenol red indicator to yellow-orange from red colour. The colour changes within ten minutes for bacteria producing *Klebsiella pneumoniae* Carbapenemases and some more time is needed to detect other types of Carbapenemases.[40]

3.18.2 TESTS FOR DETECTION OF SPECIFIC TYPES OF CARBAPENEMASES:

According to CLSI 2016, Modified Hodge test is used in phenotypic confirmation of all types of Carbapenemases. Combined disc test and EDTA disc synergy test are phenotypic tests for Metallobetalactamases. Amp C disc test is done to detect Ambler class C Cephalosporinases. [41]

3.18.2.1 MODIFIED HODGE TEST (MHT):

MHT is the phenotypic test used for confirmation of production of Carbapenemases by gram negative bacilli according to CLSI. It is most sensitive for detection of KPC Carbapenemases and has varying sensitivity for other types of Carbapenemases.[37]It is not done as a routine, but done for epidemiological purposes.[37] It can also give false positive results with ESBL and Amp C producers.[37]It is based on the principle that Carbapenemase produced by the test strain is sufficient to inhibit the action of Carbapenem antibiotic thereby allowing the growth of ATCC *E.coli* 25922 near strain that is tested, producing clover leaf zone.[37]

3.18.2.2 EDTA DISK SYNERGY TEST (EDS):

This test is done to detect MBLs in Carbapenem resistant bacteria. It is based on the principle that Metallobetalactamases require metal ions (Zinc) for hydrolysis of Carbapenems.[41] Chelators like EDTA chelates the zinc ions making it unavailable for hydrolysing action, thus producing an enhanced zone of inhibition.[41] The test has a sensitivity of 95% and specificity of 92% to detect MBL producers according to the study by Young et al. A study by Lee et al. has proved 100% sensitivity and specificity. MBLs are better detected by the EDS test than with MHT.[41]

3.18.2.3 COMBINED DISC TEST (CDT):

In combined disc test, as the name suggests, two discs are used. One disc contains the Carbapenem antibiotic and the other disc contains the antibiotic with an inhibitory agent. The inhibitor that is used can be Phenyl boronic acid (PBA) or Ethylene diamine tetra acetic acid (EDTA) depending on the type of Carbapenemase that is desired to be detected. The former is used to detect *Klebsiella pneumoniae* Carbapenemases and the latter is used to detect Metallobetalactamases. These methods are even more accurate in identifying the type of Carbapenemase.[41]

3.18.2.4 E TEST – DETECTION OF METALLOBETALACTAMASES:

Carbapenem antibiotic like Imipenem and the antibiotic with an inhibitor (Ethylene diamine tetra acetic acid) can be used in the form of E-strip with the antibiotic at one end of the strip and the drug with inhibitor at the other end, for the detection of Metallobetalactamase production by bacterial strains, on a Muller Hinton agar plate. When the MIC is decreased by three fold or more at the EDTA end, the isolate is identified as a Metallobetalactamase producer. In spite of some false negative results, this method can be used because it has a high sensitivity and specificity.

3.18.2.5 AMP C DISK TEST:

This test helps in the detection of AmpC producers, which contribute to Carbapenem resistance. The sensitivity and specificity of this test in detecting Ambler class C β-lactamases are 100% and 98% respectively.[41]

3.18.3 CONFIRMATORY TESTS FOR CARBAPENEMASES:

3.18.3.1 GENOTYPIC METHODS:

PCR is the reliable and most satisfactory method for detecting genes coding for Carbapenem resistance. But it cannot be done routinely because it is costly.[10] Genotyping is mainly of epidemiological value.[10]

3.18.3.2 DNA HYBRIDIZATION:

DNA hybridization is being used for the detection of resistance determining genes or the genes that are responsible for resistance to Carbapenems. Special hybridization techniques can detect the presence of gene and the location of the gene as well, that is, whether the gene is in the chromosome or in the plasmid.[10]

3.18.3.3 POLYMERASE CHAIN REACTION (PCR):

This test is made the most specific by complete gene sequencing and use of primers that are specific for the resistant gene that is to be detected. It is also highly sensitive. This is the gold standard test for identifying Carbapenem resistance determining genes.[10] Bio fire and Nanosphere are other molecular techniques to detect Carbapenemases.[10]

3.19 ANTIBIOTICS TO TREAT CRE INFECTIONS:

Studies were carried out in critically ill patients with *CRE* infections with single drug treatment and combination therapy with Tigecycline, Colistin and Carbapenems and have concluded that mortality of critically ill patients were lower when treated with combination therapy than with monotherapy[43].

COLISTIN:

Polymyxin B and Colistin are the agents which show maximum invitro activity against *CRE*.[48] Higher doses, may cause nephrotoxicity and drug resistance can occur with monotherapy. Patients treated with antibiotics in combination with Colistin survived better.[43]

TIGECYCLINE:

Though many CRE show good invitro activity against Tigecycline; there is increasing resistance to Tigecycline with monotherapy.[49]Tigecycline treatment may be very beneficial and efficacious when we use the drug in doses higher than usual and when Tigecycline is used in combination with another antibiotic for serious CRE infections.[44]

FOSFOMYCIN:

Oral fosfomycin has been successfully used for treating UTIs as approved by FDA.[44]

AMINOGLYCOSIDES:

Aminoglycoside treatment was found to clear resistant organisms better when compared with either Polymyxin B or Tigecycline[44]. The combination of a Carbapenem and an Aminoglycoside had the lowest mortality rate.[44]

3.19.1 COMBINATION THERAPY:

Decreased mortality, Potential synergistic effects and reduction of emerging resistance are the benefits of combination therapy. However, the risk for development of *Clostridium difficile* infection is high. The risk of adverse effects like nephrotoxicity is also increased[45]. The benefits are more than the risks and hence combination therapy is recommended for CRE.[45]

3.20 EMERGING NEWER TREATMENT OPTIONS FOR *CRE* INFECTIONS:

Ceftazidime-Avibactam is a combination drug that was approved in 2015 by FDA for the treatment of infections of the abdomen and urinary tract that were highly complicated.[44]It has activity against all Carbapenemases except Metallo-β-lactamases[44]. Patient receiving Ceftazidime-Avibactam can experience constipation, seizures, vomiting and hypersensitivity as adverse effects. [46] The drug is under phase III trial.[44]

The burden of resistance to antibiotics is going on increasing day by day in gram negative bacteria. Organisms resistant to Carbapenems are on rapid raise, especially the members of *Enterobacteriaceae*. There are very limited drugs/ antibiotics that can successfully treat these infections. So correct dose of antibiotics, that too under true / absolute indications alone can help prevent further spread of resistance to antibiotics.[44]

4. MATERIALS AND METHODS

- **Design of the study:** Hospital based Prospective cross sectional study.
- **Study period:** October 2014 to December 2015 (1 year and 3 months).
- **Setting :** Department of Microbiology of tertiary care hospital.
- **Material for the study:** All gram negative bacilli isolated from heterogenous clinical samples like urine, sputum, blood, pus, and other body fluids received in our laboratory for bacterial culture and identified as members of *Enterobacteriaceae* by standard laboratory tests were included in the study.
- **Inclusion criteria:** Gram negative bacilli that are isolated from heterogenous clinical samples and identified as members of *Enterobacteriaceae* showing resistance and intermediate sensitivity to Carbapenems by standard laboratory tests.
- **Exclusion criteria:** Gram negative bacilli that are isolated from heterogenous clinical samples and identified as non *Enterobacteriaceae* and *Enterobacteriaceae* sensitive to Carbapenems by standard laboratory tests.
- **Data entry:** All data were entered into a proforma sheet.
- **Data analysis:** Data was analysed manually by descriptive statistics.
- **Informed consent:** Obtained.
- **Ethical committee approval:** Enclosed.

4.1 PATIENT HISTORY

Age, sex, demographic details, Clinical condition, duration of illness, antibiotic history and other relevant history were collected from the laboratory request forms.

4.2 COLLECTION OF CLINICAL SAMPLES:

All the CRRI, nurses and other paramedical staffs in the sample collection area were instructed to collect clinical samples from patients according to the 2015 CDC guidelines and were also advised to instruct the patients on the standard collection procedures for urine and sputum. All the clinical departments were instructed to send patient samples before administration of antibiotics, and to transport the samples to the laboratory as and when they were collected, in appropriate transport media, if needed without delay.

4.3 SAMPLE PROCESSING:

- Gram staining/ direct microscopic examination of the samples were done appropriately for various clinical samples.
- Urine samples were inoculated on to Nutrient and CLED agar, Sputum samples onto Nutrient, blood, Chocolate and MacConkey agar. Blood, Pus and Body fluids onto Nutrient, MacConkey and Blood agar. Stool samples onto MacConkey and SS agar (*Salmonella Shigella* agar).
- The culture plates were incubated overnight at 37^{O}C.
- Next day the colony characters were observed and the organisms were identified as lactose fermenters and non lactose fermenters.

- Gram staining was done on the colonies and those colonies identified as gram negative bacilli were further subjected to catalase, oxidase and motility tests initially.
- All gram negative bacilli which were catalase positive and oxidase negative were characterised as *Enterobacteriaceae*.
- The isolates characterised as *Enterobacteriaceae* were subjected to various biochemical tests like tests for indole production, citrate utilisation, urease production, nitrate reduction, Mannitol fermentation and inoculation into TSI agar medium for identification of Genera and species.

4.4 ANTIMICROBIAL SUSCEPTIBILITY TESTING:

Antimicrobial susceptibility testing was done by Kirby Bauer disc diffusion assay for the standard antibacterial drugs used in the *Enterobacteriaceae* panel according to 2016 CLSI guidelines (Ampicillin10µg, Gentamycin10µg, Amikacin10µg, Cefotaxime10µg, Cefoxitin30µg, Cotrimoxazole25µg, Ciprofloxacin5µg, Piperacillin-Tazobactam100/10µg, Aztreonam 30µg, Nitrofurantoin300µg (Only for urinary isolates) and Meropenem10 µg) using *E.coli* ATCC 25922 as Quality control.[37]

Procedure (**Kirby Bauer disc diffusion method**):

- The test organism was inoculated into peptone water and incubated such that it matches a turbidity of 0.5% Mc Farland standards and it was inoculated as a lawn over Muller Hinton agar (MHA) plate. Antibiotic discs were placed over the lawn – 6 discs in a 90 mm plate[37]

- The plate was incubated at 37ºC for 24 hrs and the diameters of zones of inhibition to various antibiotics were read and the organisms were categorised as Sensitive, Intermediate and Resistant to each antibiotic according to current CLSI guidelines.[37]
- The bacterial strains that were intermediate or resistant to Meropenem were considered presumptive Carbapenemase producers and were taken for further testing.

Table 9 Disc diffusion - CLSI guidelines for Carbapenems for *Enterobacteriaceae*

Antibiotic	S (mm)	I (mm)	R (mm)
Meropenem	≥23	20-22	≤19
Imipenem	≥23	20-22	≤19

4.5 TESTS FOR CARBAPENEMASE PRODUCTION:

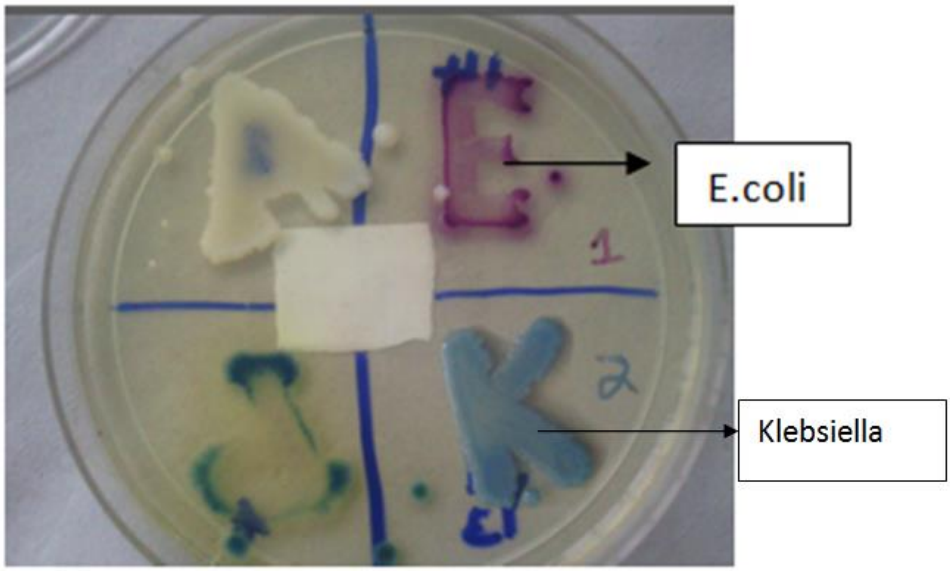
The presumptive Carbapenemase producers were subjected to various tests to confirm Carbapenemase production, which included growth on KPC CHROME agar, Modified Hodge test, EDTA disc synergy test, Combined disc test and Amp C disc test.

4.5.1 GROWTH ON KPC CHROME AGAR:

KPC CHROM agar preparation powder was obtained from Himedia. The instruction manual was provided according to which the media was prepared and stored, preferably in a light free area at a temperature of 4°C .[38]

- The suspected Carbapenemase producing *Enterobacteriaceae(CRE)* isolates were inoculated onto KPC chrome agar and incubated over night as for routine bacterial cultures.[38]
- As per the manufacturer's instruction, the isolates taking colours were identified as Carbapenemase producers.[38] (For example. *E.coli* and *Citobacter* appeared magenta, *Klebsiella* and *Enterobacter* appeared metallic blue as shown in Figure 3.

Figure 3 Growth of Gram negative bacilli on KPC CHROM agar

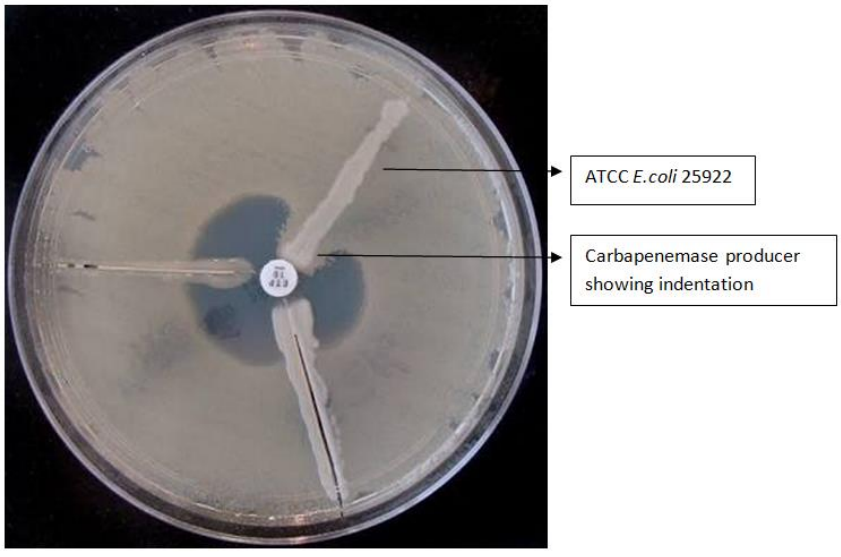


The results were recorded and tabulated.

4.5.2 MODIFIED HODGE TEST:

- An overnight culture suspension of ATCC *E.coli* 25922 was prepared in peptone water, matched to 0.5 McFarland turbidity standards, diluted to one in ten and inoculated as lawn culture over a 90mm MHA plate as for disk diffusion.[37]
- After waiting for 3-5 mins for drying, a Meropenem disc was placed at the centre of the plate.
- Using a loop which can deliver 10 microlitre, the test organism was taken and streak inoculated from the disk edge towards all four directions. 4 isolates were tested in a plate with a single Meropenem disc. The plate was incubated at 35 ± 2^0C for 16-20 hrs.
- The plates were examined the next day for enhanced growth around the test organism and the zone of inhibition giving a clover leaf appearance, which was indicative of Carbapenemase production[37] as shown in Figure4.The results were recorded and tabulated.

Figure 4 Modified Hodge test



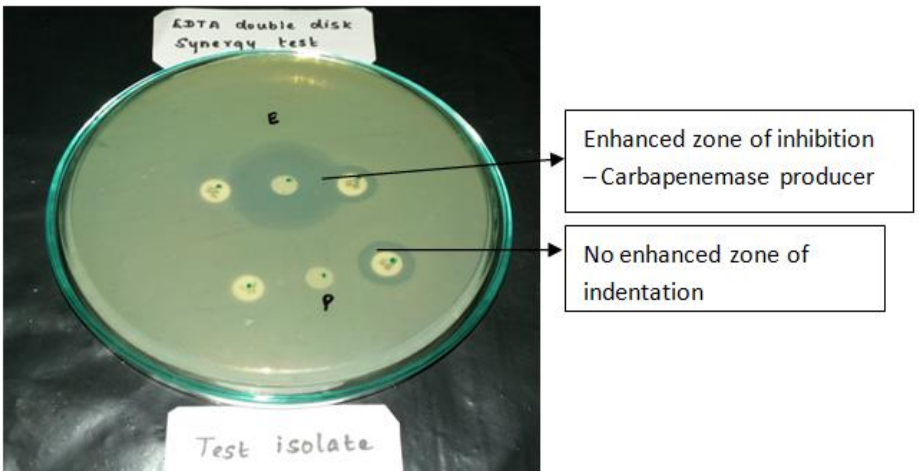
4.5.3 EDTA DISK SYNERGY TEST:

- All the meropenem resistant isolates were simultaneously tested with ceftazidime and Meropenem to detect metallo-β-lactamase production.[41]

- A culture suspension of the test isolate that had been incubated in broth overnight was taken, matched to 0.5 McFarland turbid standards and inoculated as a lawn on Muller Hinton Agar plate. A Meropenem disc with a potency of 10 µg and a Ceftazidime disk with a potency of 30 µg (HIMEDIA) were kept on the agar plate over the lawn. EDTA disc was kept in the middle of the two discs with an edge to edge distance of 10mm from both the discs.

- The plate was incubated overnight at 37^{o} C and an expansion in the zone of inhibition between the EDTA disc and either of the other two disks as shown in figure 5 was interpreted as positive for MBL production[41]. The results were recorded and tabulated.

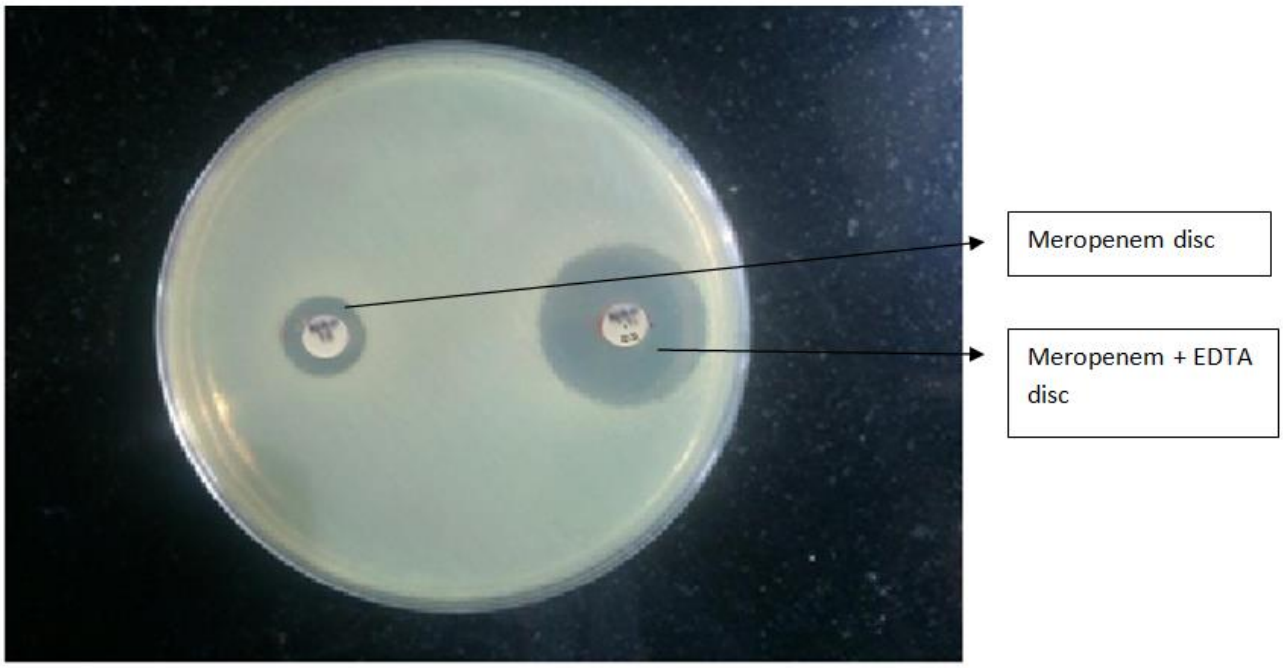
Figure 5 EDTA disc synergy test



4.5.4 COMBINED DISC TEST (CDT):

- On a MHA plate, the test organism adjusted to 0.5 McFarland standards from an overnight culture suspension was inoculated as a lawn.
- One disc with Meropenem and the other disc containing Meropenem with EDTA were placed over the lawn[41]. A 4-5mm increase in zone diameter in the Meropenem- EDTA disc compared to Meropenem disc alone was taken as positive for Metallobetalactamase production[41] which is shown in Figure 6. The results were recorded and tabulated.

Figure 6 Combined disc test



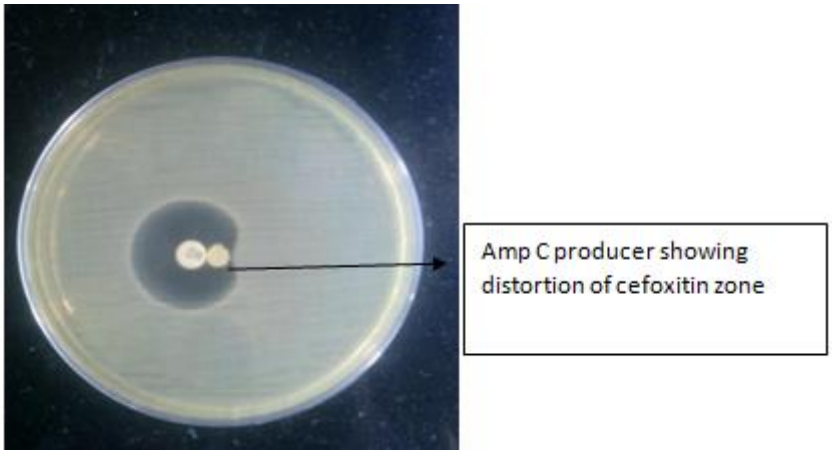
4.5.5 TEST TO IDENTIFY AMP C PRODUCERS:

4.5.5.1 AMP C DISK TEST:

All the Meropenem resistant strains were subjected to Amp C disk test to detect the production of Ambler class C β-lactamase.[41]

- An overnight culture suspension of ATCC *E.coli* 25922 was prepared in peptone water, matched to 0.5 McFarland turbidity standards and inoculated as lawn culture over a 90mm MHA plate as for routine disk diffusion procedure.[37]
- A Cefoxitin disk with a potency of 30 microgram was placed over the lawn.
- An empty disk moistened with sterile saline and inoculated with the test organism was placed at the vicinity of the Cefoxitin disk almost touching it. The culture plate was kept in the incubator for overnight incubation at 37^{o} C.[41]
- Blunting of the zone of inhibition of cefoxitin near the test strain inoculated disc was taken as indicative of the strain being a producer of Ambler class C betalactamase, as shown in Fig7. [41]
- The results were recorded and tabulated.

Figure 7 Amp C disc test



4.6 TESTING THE CARBAPENEM RESISTANT ISOLATES FOR SUSCEPTIBILITY TO TIGECYCLINE AND COLISTIN:

Susceptibility of the Carbapenem resistant isolates to tigecycline[56] and colistin[57] was done by disc diffusion method according to EUCAST and guidelines of the study by Irene et al.

Procedure (**Kirby Bauer disc diffusion method**):

- The test organism was inoculated into peptone water and incubated such that it matches 0.5% Mc Farland turbidity and it was inoculated as lawn culture over Muller Hinton agar plate.
- Tigecycline 15µg and colistin 5 µg antibiotic discs were placed over the lawn[47]
- The plate was incubated at 37ºC for 24 hrs and the next day the diameters of zones of inhibition were read and the organisms were categorised as Sensitive, Intermediate and Resistant accordingly.[47,48]

Table 10 Disc diffusion – EUCAST and Irene et al guidelines for Tigecycline and colistin respectively for *Enterobacteriaceae*

Antibiotic	S	I	R
Tigecycline[47]	≥18	15-18	≤14
Colistin[48]	≥14	12-13	≤11

4.7 GENOTYPING:

25 CRE isolates with odd numbers, which included 16 *Klebsiella spp.,* 7 *E.coli* and 2 *Proteus spp*., were randomly chosen and were subjected to Genotyping to identify the genes *bla*NDM-1 and *bla*OXA51 by Multiplex PCR.

- Bacterial DNA was purified and DNA was extracted from all the strains, mixed with specific primers and placed into the PCR machine.
- Multiplex PCR was carried out in order to detect *bla*NDM-1 and *bla*OXA-51genes.
- The steps of PCR are initial denaturation at 95°C for 5 mins, followed by 35 cycles of denaturation at 94°C for 30 sec, which is inturn followed by annealing at 58°C for 30 sec and extension at 72°C for 30 sec, and finally an extension at 72°C for 5 min.
- The products that were amplified were subjected to electrophoresis at 50 V in 2% agarose gel stained with ethidium bromide and the bands were visualized under Ultraviolet light.

The resistant genes were thus identified.

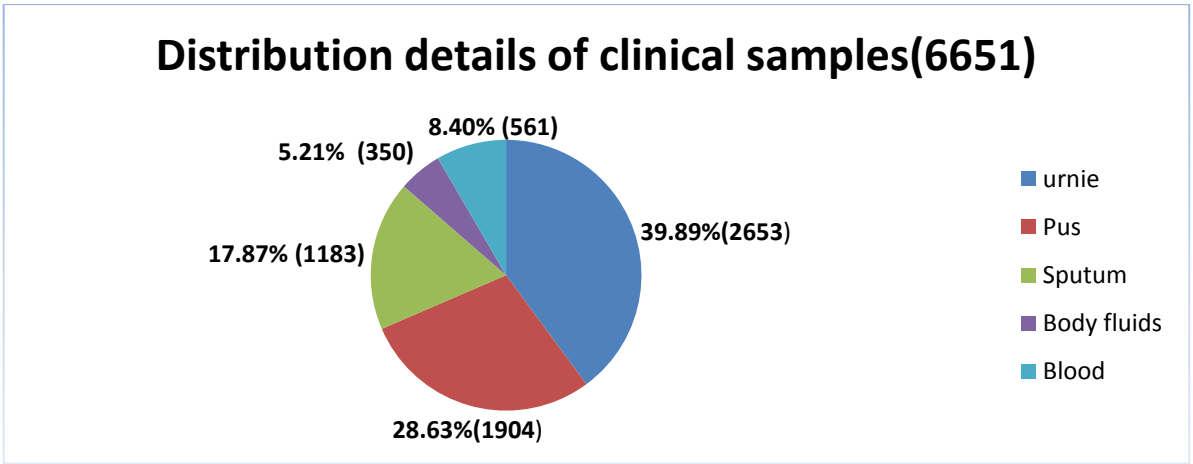
5. RESULTS

The study, **"PREVALENCE, PHENOTYPING AND MOLECULAR DETECTION OF *bla*NDM-1 and *bla*OXA-51 GENES IN CARBAPENEMASE PRODUCING STRAINS AMONG THE CARBAPENEM RESISTANT *ENTEROBACTERIACEAE*"** was carried out in the department of Microbiology, Chennai Medical college Hospital and research centre, Trichy and the results have been discussed with reference to parameters like age, gender, inpatient/outpatient details and co-morbid conditions.

5.1 SAMPLES RECEIVED IN OUR LABORATORY DURING THE PERIOD OF STUDY:

The distribution details for all the **6651** clinical samples received in our laboratory during the fifteen months (October 2014 to December 2015) study period is depicted in Figure 8, given below.

Figure 8 Distribution details of samples received during the study period (Oct 2014 to Dec 2015)



Figures in parenthesis indicate the number of each clinical sample received.

5.2 BACTERIAL ISOLATES FROM HETEROGENOUS CLINICAL SAMPLES:

The total number of bacteria that were isolated during the study period was 4423(66.5%) out of 6651 samples that were processed. The details of the number of Gram positive cocci, Gram negative bacilli including *Enterobacteriaceae* and *Non-fermenters*, contaminants and skin commensals isolated during the period of our study are provided in Table 11.

Table 11 Total and Sample-wise isolation of bacteria during the study period

Nature of bacteria	Pus	Sputum	Urine	Blood	Body fluids	Total	%*
GPC*	240	15	11	72	9	347	5.3
GNB*	799	585	542	60	52	2038	30.6
NG/Contaminant/ Commensal	865	583	2100	429	289	4266	64.1
Total	1904 (28.63)	1183 (17.87)	2653 (39.89)	561 (8.4)	350 (5.21)	6651 (100)	100

*NG- No growth; GPC-Gram positive cocci; GNB-Gram negative bacilli; Figures in parenthesis indicate percentage.

Out of 4423 bacteria that were isolated, 2038 (30.6%) were Gram negative bacilli, which included 1421 (69.72%) *Enterobacteriaceae* and 617 (30.28%) nonfermenters. The distribution details of the same have been furnished in Table12.

Table 12 Distribution details of all Gram negative bacilli isolated during the study period

Nature of bacteria	Pus (39.2%)	Sputum (26.79)	Urine (24.76)	Blood (4.34)	Body fluids (2.55)	Total	(%)
Enterobacteriaceae	589	285	482	33	32	1421	69.72
Non-fermenters	210	300	60	27	20	617	30.28
Total	799	585	542	60	52	2038	100

5.3 SAMPLEWISE ISOLATION OF DIFFERENT MEMBERS OF *ENTEROBACTERIACEAE*:

Sample wise isolation of *Enterobacteriaceae* has been depicted in Table 13.

Table 13 Sample-wise isolation of different members of Enterobacteriaceae

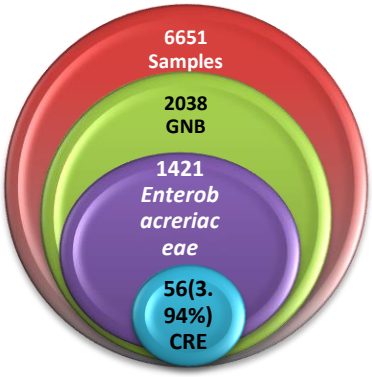
Enterobacteriaceae isolated	Pus	Sputum	Urine	Blood	Body fluids	Total	%*
E.coli	213 (36.2)	48(16.8)	354(73.4)	9(27.3)	10(31.2)	634	44.6
Klebsiella spp.,	243 (*41.2*)	191(67.1)	84(17.4)	18(54.5)	21(65.6)	557	39.2
Proteus spp.,	93 (15.8)	28(9.8)	20(4.2)	-	-	141	9.9
Citrobacter spp.,	18(3.05)	18(6.3)	12(2.5)	-	1(3.2)	49	3.4
Serratia spp.,	15(2.6)	-	-	-	-	15	1.1
Providencia spp.,	7(1.2)	-	12(2.5)	-	-	19	1.4
Salmonella	-	-	-	6(18.2)	-	6	0.4
Total	589 (41.4)	285 (20.1)	482 (33.9)	33 (2.4)	32 (2.2)	1421	100

*- Figures in parenthesis indicate percentage.

5.4 DISTRIBUTION OF CARBAPENEM RESISTANT *ENTEROBACTERIACEAE (CRE)*:

Out of 1421 *Enterobacteria*, Carbapenem resistance was observed in 56(3.94%) isolates that consisted of 25 *Klebsiella spp.*, 21 *E.coli*, 8 *Proteus* spp., and 2 *Citrobacter spp.*, on disc diffusion assay using Meropenem according to CLSI guidelines 2016.

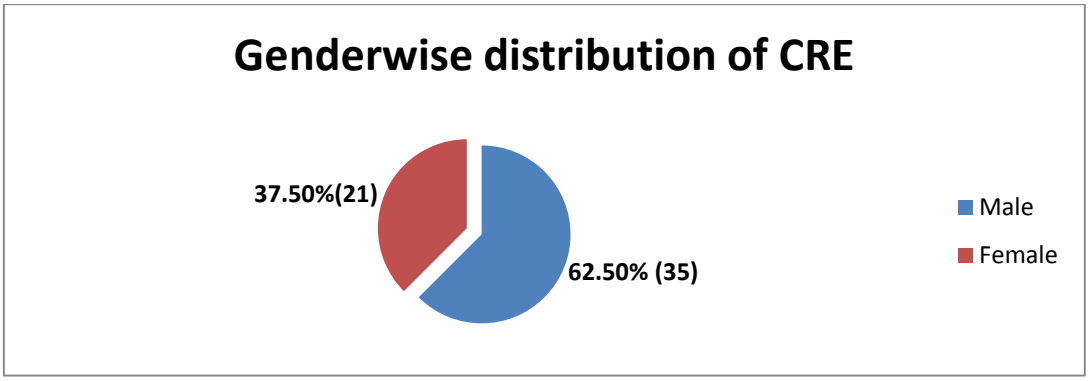
Figure 9 Total number of bacterial isolates including *CRE*



5.5 GENDERWISE DISTRIBUTION OF *CRE*:

Out of 56 isolates, 35(62.5%) were from males and 21(37.5%) were from females. The distribution of *CRE* in relation to gender is shown in Figure 10.

Figure 10 Gender-wise distribution of *CRE*



Figures in parenthesis indicate the number of isolates from each gender.

5.6 AGE AND GENDERWISE DISTRIBUTION OF *CRE:*

The *CRE* isolates were more from males and it was significant. Similarly the *CRE* were more from the 51 and above age group. The youngest patient from whom the *CRE* was isolated belongs to the 11-20 age group and the oldest to the above 60 age group. The distribution of the *CRE* isolates in relation to age and gender is depicted in Table14.

Table 14 Distribution of *CRE* in relation to age and gender

Age group (years)	Gender		Total no. of isolates (%)*
	Male	Female	
1-10	0	0	0
11-20	1	0	1(1.78)
21-30	1	1	2(3.57)
31-40	3	5	8(14.28)
41-50	1	4	5(8.92)
51-60	12	7	19(33.92)
>60	17	4	21(37.5)
TOTAL	35(62.5%)	21(37.5%)	56

*-Figures in parenthesis indicate percentage.

The *CRE* were observed predominantly in the above 60 age group.

5.7 DISTRIBUTION OF CARBAPENEM RESISTANCE IN DIFFERENT MEMBERS OF *ENTEROBACTERIACEAE*:

Table 15 depicts the distribution of Carbapenem resistance in various members of *Enterobacteriaceae*.

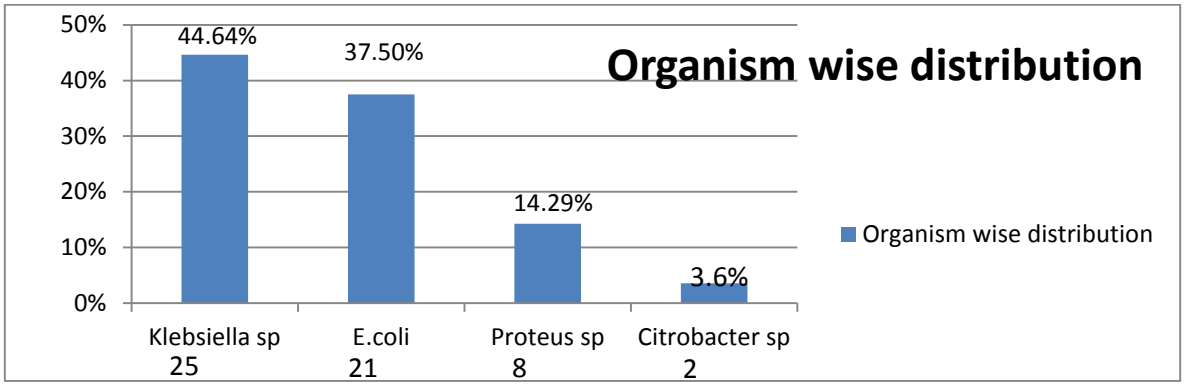
Table 15 Distribution of Carbapenem resistance in different members of *Enterobacteriaceae*

***Enterobacteriaceae* member**	**Total no**	**Meropenem resistance (%)**
E.coli	634	21(3.31)
Klebsiella spp.,	557	25(4.49)
Proteus spp.,	141	8(5.67)
Citrobacter spp.,	49	2(4.08)
Total	1381	56

5.8 DISTRIBUTION OF VARIOUS MEMBERS OF CARBAPENEM RESISTANT *ENTEROBACTERIACEAE*:

Out of 56 *CRE* isolates, 25(44.64%) were *Klebsiella spp.*, which included 24 *Klebsiella pneumoniae* and 1 *Klebsiella oxytoca*, 21(37.5%) were *E.coli*, 8(14.29%) were *Proteus spp., which included 4 Proteus mirabilis and 4 Proteus vulgaris* and 2(3.6%) were *Citrobacter spp.,* comprising 1 *Citrobacter koseri* and 1 *Citrobacter freundii*. The details have been furnished in Figure11.

Figure 11 Distribution of *CRE* in relation to organisms



5.9 DISTRIBUTION OF CARBAPENEM RESISTANCE IN *ENTEROBACTERIACEAE* IN VARIOUS CLINICAL SAMPLES:

The distribution of Carbapenem resistance in *Enterobacteriaceae* with regard to clinical samples is provided in Table16.

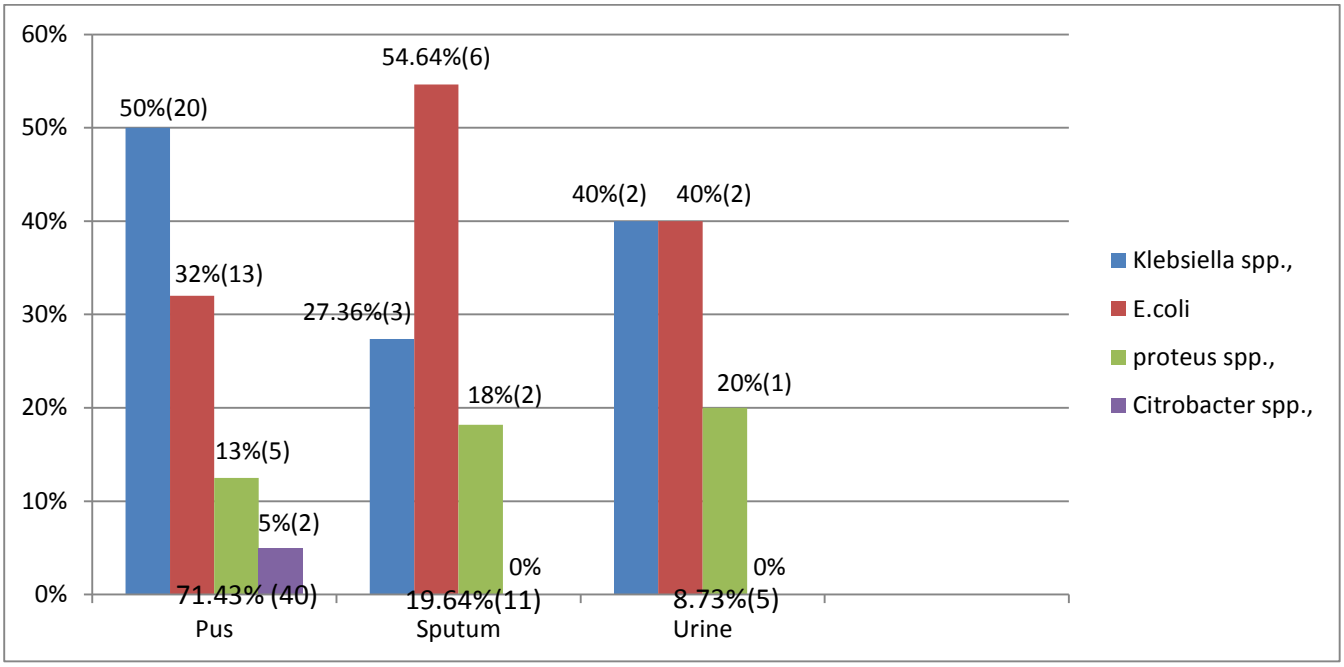
Table 16 Distribution of CRE in various clinical samples

Nature of Sample	Total no of *Enterobacteriaceae*	Carbapenem resistance n (%)
Pus	589	40(6.79)
Sputum	285	11(3.86)
Urine	482	5(1.04)
Blood	33	-
Body fluids	32	-
Total	1421	56

5.10 DISTRIBUTION OF DIFFERENT MEMBERS OF CARBAPENEM RESISTANT *ENTEROBACTERIACEAE* IN RELATION TO VARIOUS CLINICAL SAMPLES:

The distribution of CRE in relation to organisms and clinical samples is described in Figure12.

Figure 12 Distribution of various members of *CRE* in relation to clinical samples



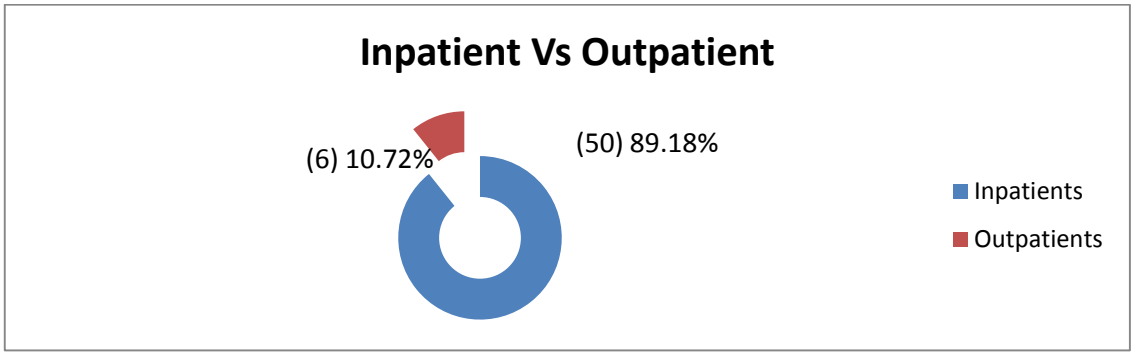
Figures in parenthesis indicate the number of each bacterial isolate.

CRE were more in pus samples probably due to indiscriminate antibiotic use. No *CRE* was isolated from blood and body fluids.

5.11 DISTRIBUTION OF *CRE* IN RELATION TO IN-PATIENTS AND OUT-PATIENTS:

Figure13 represents the percentage of in and out-patients with *CRE* infections.

Figure 13 Distribution of *CRE* in relation to In-patients and out-patients



Figures in parenthesis indicate the number of in-patients and out-patients.

5.12 DISTRIBUTION OF CRE IN RELATION TO WARDS:

As expected the isolates were more from surgical wards (40/56) making 71.43% as against 16/56 (28.57%) from medical wards and the distribution is statistically significant as shown in Table17

Table 17 Distribution of *CRE* in relation to wards (56 No.)

S No	Ward	*E.coli*	*Klebsiella spp.,*	*Citrobacter spp.,*	*Proteus spp.,*	Total	%
1	Surgery*	7	13	2	4	26	46.43
2	Orthopaedics*	2	2	-	-	4	7.14
3	Burns*	1	-	-	1	2	3.57
4	OG*	3	2	-	-	5	8.93
5	Casualty*	1	1	-	1	3	5.36
6	Chest medicine**	1	3	-	1	5	8.93
7	ICU**	1	1	-	1	3	5.36
8	Medicine**	4	2	-	-	6	10.71
9	Dermatology**	1	1	-	-	2	3.57
	Total	**21**	**25**	**2**	**8**	56	100

* - Surgical wards; **- Medical wards

5.13 DISTRIBUTION OF CO-MORBID CONDITIONS AMONG THE CASES WITH *CRE* INFECTIONS:

A total of 35 out of 56 (69.64%) patients who harboured CRE isolates had one or the other co-morbid condition and the details are furnished in Table18.

Table 18 Co-morbid conditions associated with CRE infections

S No	Co-morbid condition	Number (%)*
1.	Type 2 Diabetes Mellitus	24 (42.86)
2.	Pulmonary Tuberculosis(PT)/ Post PT	3 (5.36)
3.	Systemic Hypertension	3 (5.36)
4.	Urinary Catheterisation	8 (14.27)
5.	Chronic Kidney Disease	1 (1.7)
6.	No co-morbid condition	17 (30.36)
Total		56

*- Figures in parenthesis indicate percentage.

Co-morbid conditions predispose to delayed response to treatment of the underlying infection, requiring prolonged antibiotic therapy which results in drug resistance.

5.14 ANTIBIOTIC SENSITIVITY PATTERN OF VARIOUS *CRE:*

5.14.1 SENSITIVITY OF THE *CRE* ISOLATES TO *ENTEROBACTERIACEAE* PANEL OF DRUGS ACCORDING TO 2016 CLSI GUIDELINES:

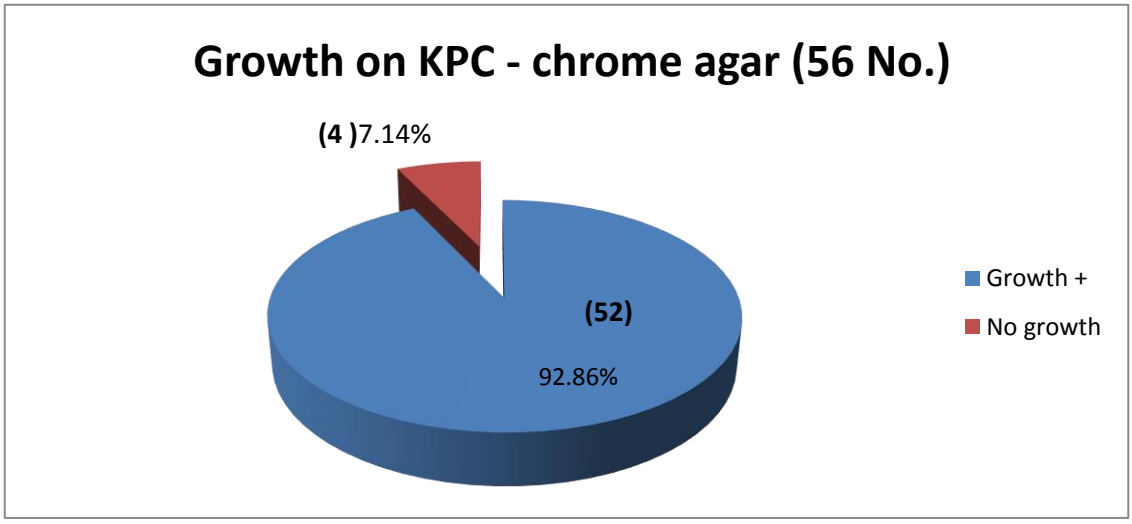
Six out of 56 *CRE* isolates (16.1%) were sensitive to Gentamycin, which included 2 *E.coli* and 4 *Klebsiella* spp., 15/56 CRE isolates (26.78%) were sensitive to Amikacin, which included 6 *E.coli,* 6 *Klebsiella spp.,* 2 *Proteus spp.,* and 1 *Citrobacter spp.,* 30/56 CRE isolates (53.57%) were sensitive to Cefoxitin, which included 15 *E.coli*, 4 *Proteus* spp,. and 11 *Klebsiella* spp., All the CRE isolates were found to be resistant to Ampicillin, Ciprofloxacin, Cotrimoxazole, Ceftriaxone, Cefotaxime, Cefoperazone – Sulbactam, Aztreonam, Imipenem and Meropenem according to 2016 CLSI guidelines. All the 56 (100%) isolates were found to be susceptible to Colistin and Tigecycline.

5.15 RESULTS OF THE PHENOTYPIC TESTS FOR DETECTION OF CARBAPENEMASES IN *CRE*:

5.15.1 GROWTH ON KPC CHROME AGAR:

On screening all the 56 isolates with KPC chrome agar, 52 (92.86%) grew on KPC chrome agar indicating Carbapenemase production, which has been depicted in Figure14.

Figure 14 Distribution of Growth of *CRE* on KPC chrome agar



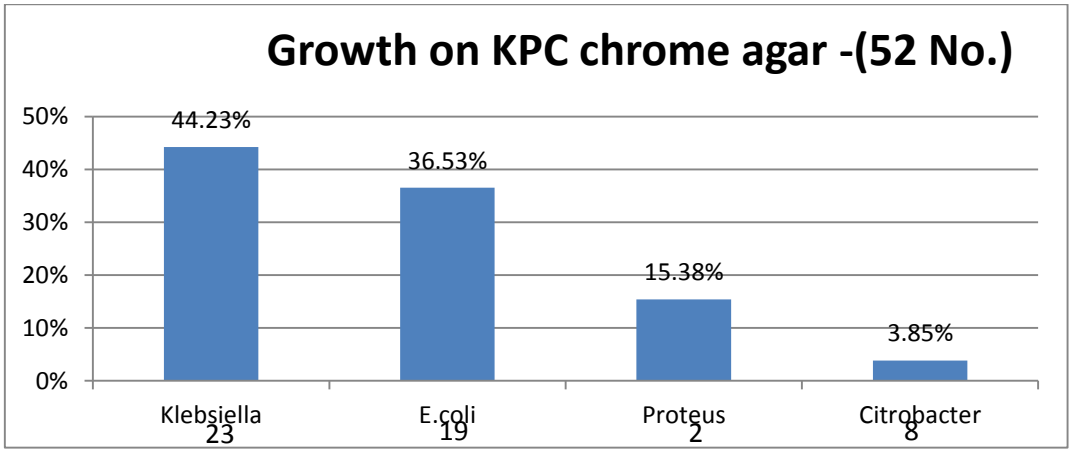
Figures in parenthesis indicate the number of positive and negative isolates.

5.15.1.1 Growth of various members of *CRE* on KPC chrome agar:

Out of the 52 isolates that were positive for growth on KPC chrome agar, 23(44.23%) were *Klebsiella spp*., followed by 19(36.53%) *E.coli*, 8(15.38%) *Proteus spp*., and 2 (3.85%) *Citrobacter spp*.,

The distribution of the organisms is given in Figure15.

Figure 15 Growth of *CRE* members on KPC chrome agar

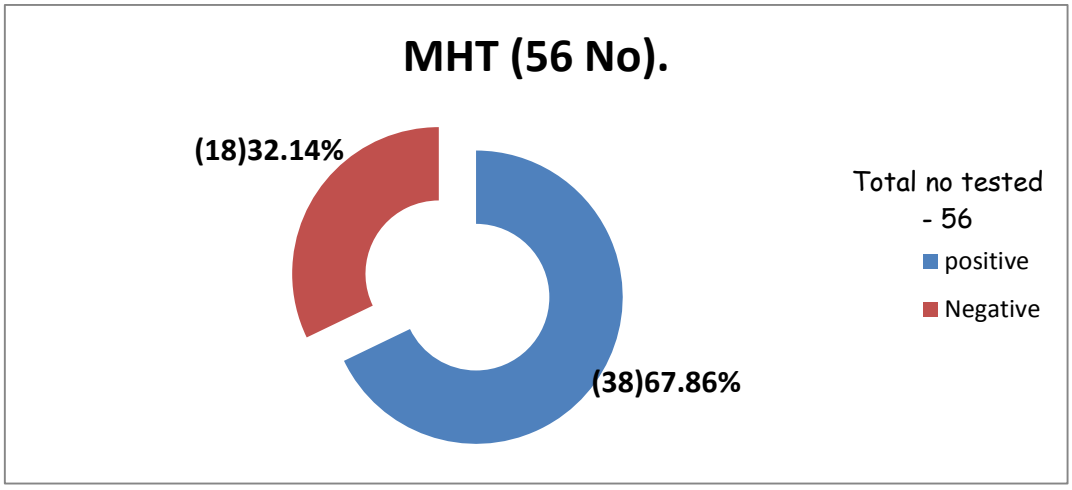


*- Figures in parenthesis indicate the number of each *CRE* isolate positive for growth on KPC chrome agar.

5.15.2. MODIFIED HODGE TEST (MHT):

Out of the 56 isolates that were tested by MHT, 38 (67.86%) were MHT positive and the remaining 18(32.14%) were negative which has been depicted in figure16.

Figure 16 MHT results of the *CRE* isolates

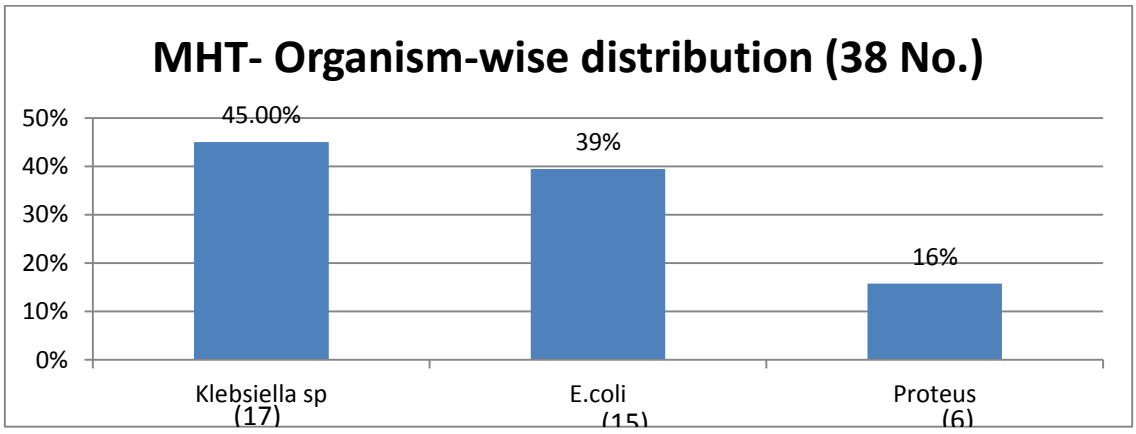


Figures in parenthesis indicate the number of MHT positive and negative isolates

5.15.2.1 Distribution of MHT positivity in relation to various members of *CRE*:

MHT positives consisted of 17 (45%) *Klebsiella spp.*, 15 (39%) *E.coli*, and 6 (16%) *Proteus spp.*, which is depicted in the figure17.

Figure 17 Distribution of MHT positivity in relation to members of *CRE*

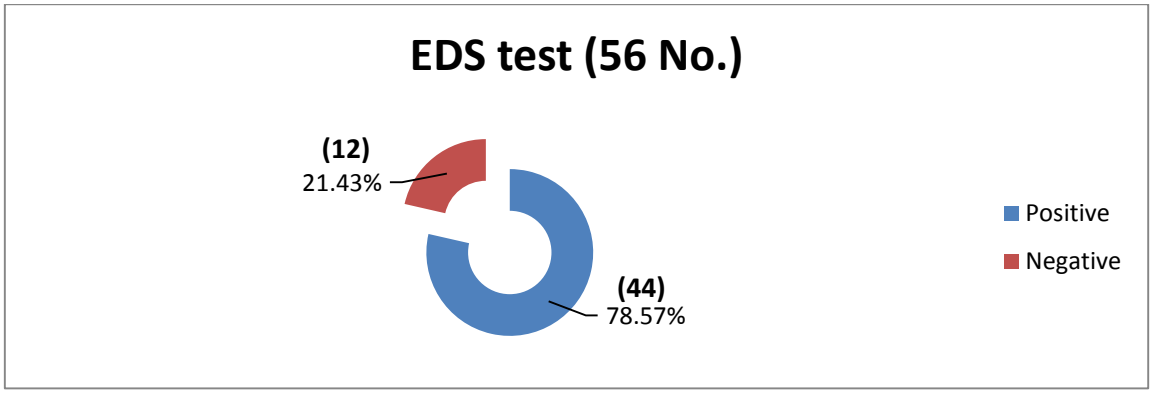


Figures in parenthesis indicate the number of each *CRE* isolate positive for MHT.

5.15.3 RESULTS OF EDTA DISC SYNERGY (EDS) TEST:

EDS test was performed on all the 56 isolates, out of which 44(78.57%) were found to be positive and 12(21.43%) were negative, which is depicted in Figure18.

Figure 18 EDS test results of *CRE* isolates

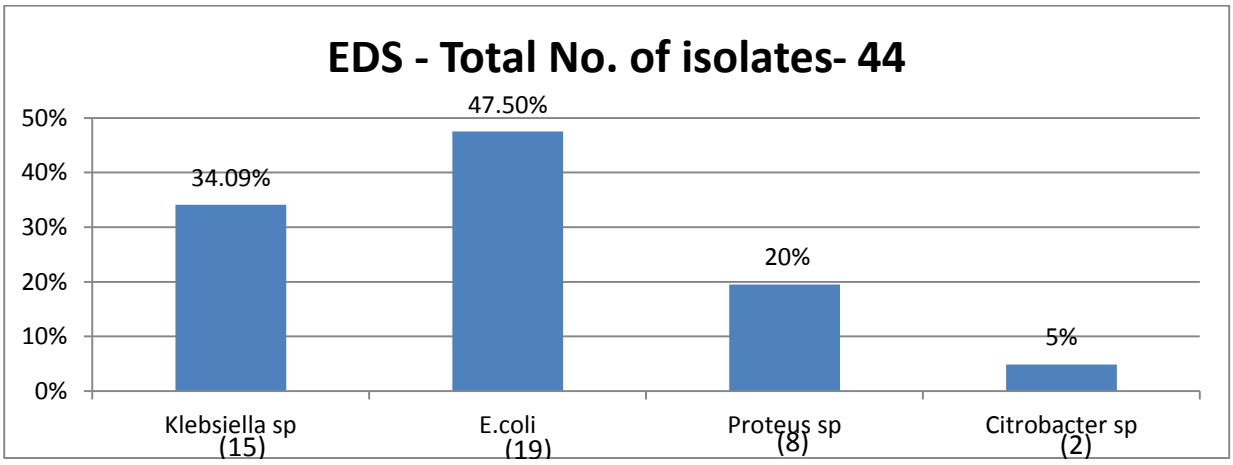


Figures in parenthesis indicate positive and negative number for EDS test.

5.15.3.1 EDS test results in relation to various members of CRE:

Among the 44 isolates positive by EDS test, 15(34.09%) were *Klebsiella spp.*, 19(47.5%) were *E.coli*, 8(20%) were *Proteus* and 2(5%) were *Citrobacter spp.*, and the same is depicted in Figure19.

Figure 19 EDS test results in relation to various members of *CRE*

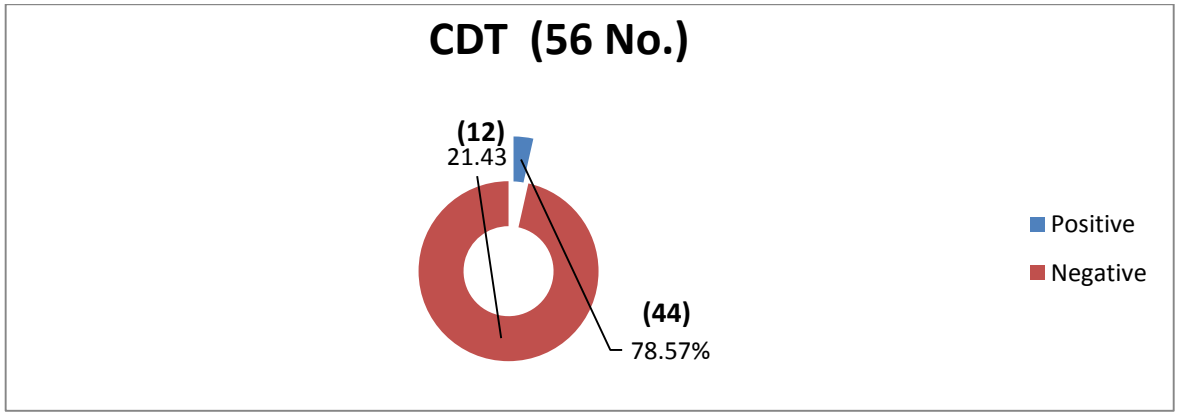


*- Figures in parenthesis indicate the number of each CRE isolate positive for EDS test.

5.15.4 RESULTS OF COMBINED DISC TEST (CDT):

Out of the 56 isolates tested, 44(78.57%) were positive and 12(21.43%) were negative for CDT (Combined disc test), which is shown in Figure 20.

Figure 20 Combined disc test (CDT) results of *CRE* isolates

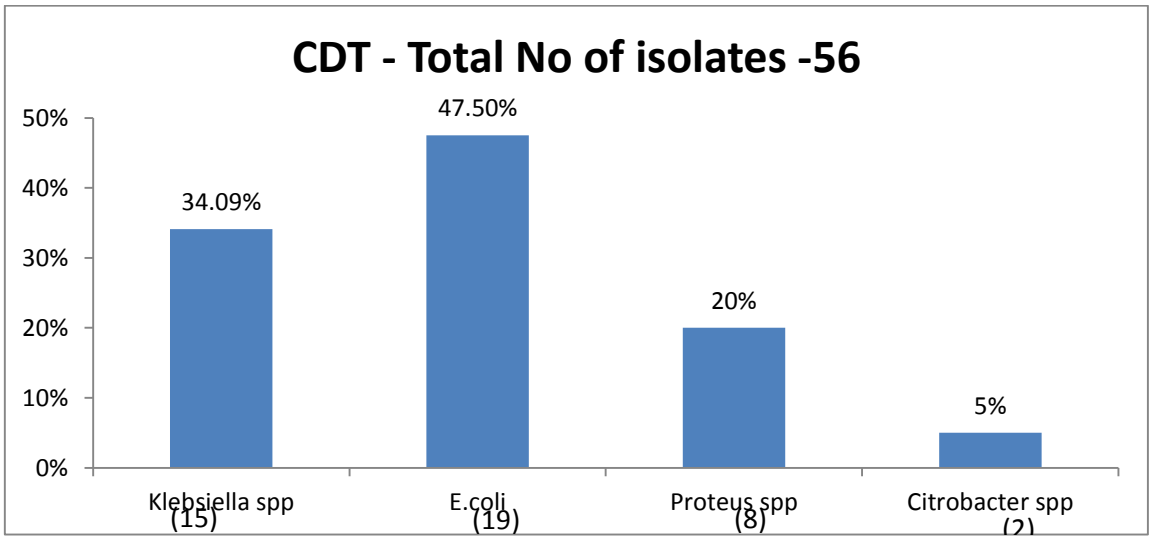


Figures in parenthesis indicate the number isolates positive and negative for CDT.

5.15.4.1 CDT results in relation to various members of *CRE*:

Among the 44 isolates that tested CDT positive 15(34.09%) were *Klebsiella spp.*, 19(47.5%) were *E.coli*, 8(20%) were *Proteus spp.*, and 2(5%) were *Citrobacter spp.*, which was similar to that of EDS test as shown in figure21.

Figure 21 CDT positivity in relation to various members of *CRE*

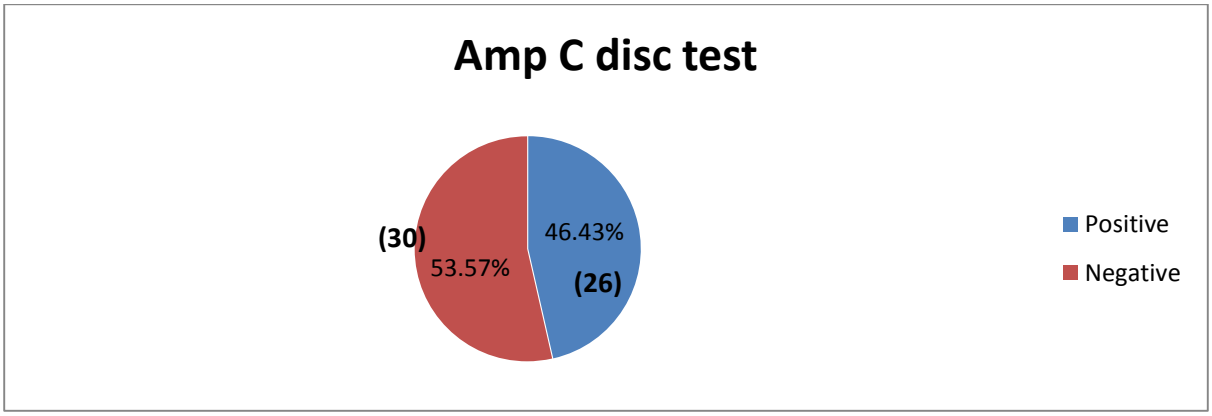


Figures in parenthesis indicate the number of each CRE isolate positive for CDT.

5.15.5 AMP C DISC TEST:

Out of 56 Carbapenem resistant isolates tested, 26(46.43%) were positive and 30 isolates (53.57%) were negative. The details are furnished in Figure 22.

Figure 22 Amp C disc test results of *CRE* isolates

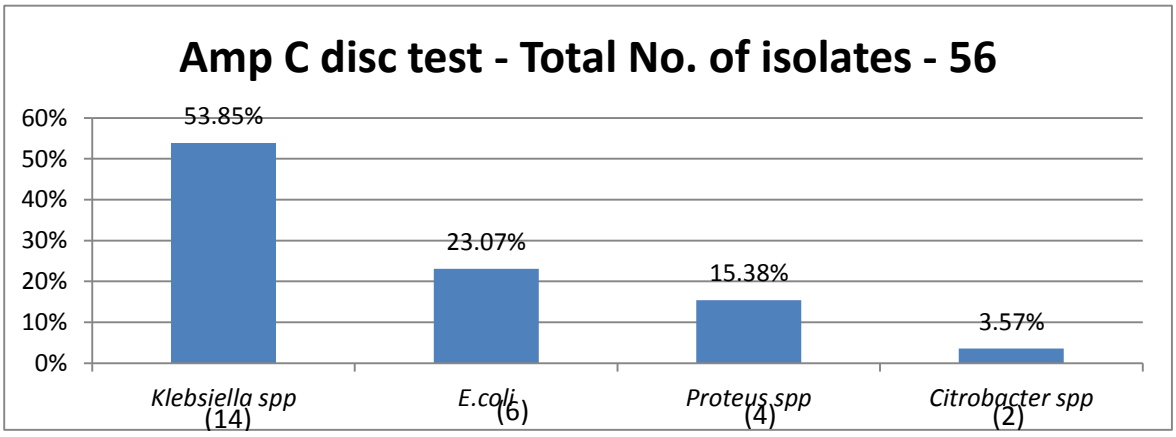


Figures in parenthesis indicate the positive and negative numbers.

5.15.5.1 Results of Amp C disc test in relation to various members of *CRE*:

The Amp C disc test was positive in 14(53.85%) *Klebsiella spp.*, 6(23.07) *E.coli*, 4(15.38%) *Proteus spp.*, and 2(3.57%) *Citrobacter spp.*, The details are given in Figure 23.

Figure 23 Amp C disc test positivity in relation to various members of *CRE*

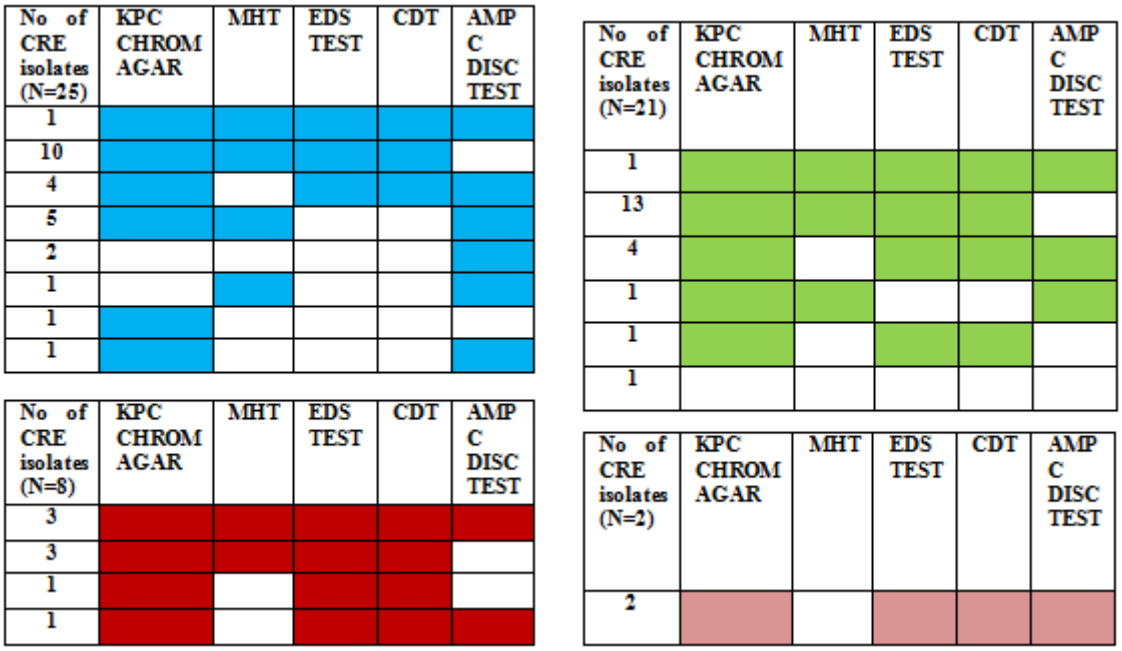


Figures in parenthesis indicate the number of each CRE isolate positive for AmpC disc test.

5.16 CRE AND PHENOTYPIC TESTS – CUMULATIVE RESULTS:

The cumulative results of the phenotypic tests performed with the CRE isolates have been depicted in Figure 24.

Figure 24 Positivity of CRE isolates for various phenotypic tests (Blue-*Klebsiella* spp., Green - *E.coli*, Brown-*Proteus spp*., Pink-*Citrobacter spp*.,)



*****-White boxes indicate negative test results. Coloured boxes indicate positive test results.

- Out of 25 Carbapenem resistant *Klebsiella* isolates, 1 was positive for all the five phenotypic tests, 10 for 4 tests except Amp C disc test, 4 for 4 tests except MHT, 5 for KPC and MHT and Amp C alone, 2 for AmpC test alone, 1 for MHT and Amp C test alone, 1 for KPC alone, 1 KPC and Amp C alone.
- One *E.coli* isolate was positive for all the five phenotypic tests. 12 for all the tests except AmpC disc test, 4 for all tests except MHT, 1 for 3 tests except

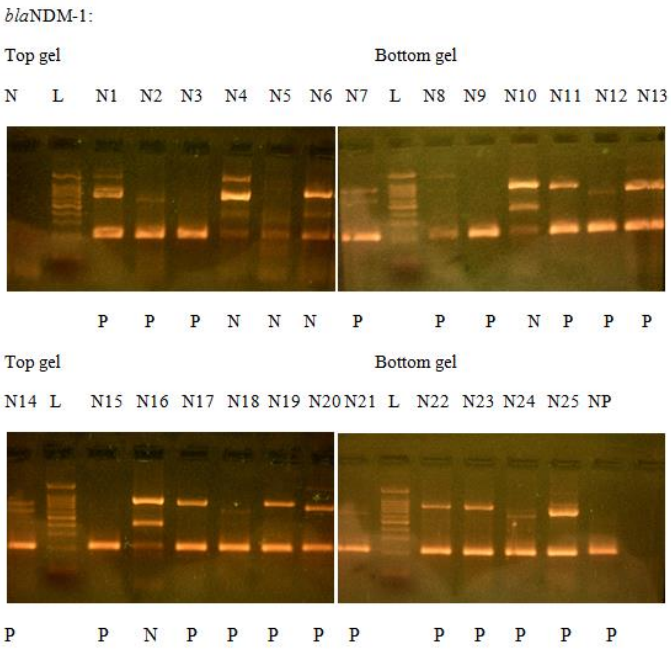
EDS and CDT, 1 for 3 tests except MHT and AmpC disc test and 1 isolate was negative for all the five tests.

- Three Carbapenem resistant *Proteus* isolates were positive for all the five phenotypic tests, 3 for four tests except Amp C disc test and 1 for all tests except MHT and 1 negative only for MHT and Amp C tests.
- Both the *Citrobacter* isolates were positive for all phenotypic tests except MHT.

5.17 RESULTS OF GENOTYPING - CRE:

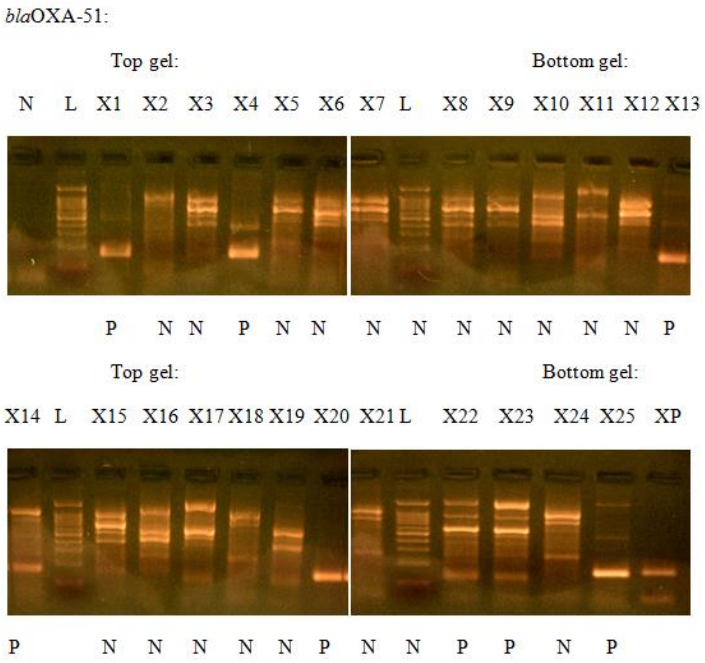
Of the 56 *CRE* isolates, genotyping was done only for 25 isolates with odd numbers due to technical limitations. Out of the 25, 21 were positive for atleast one of the target genes. Figure 25 and Figure 26 shows the results of genotyping. Table 19 shows the results of genotyping of CRE isolates for *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-51}}$ genes. The table is also furnished with the details that can help us compare the results of phenotypic and genotypic methods.

Figure 25 Results of genotyping – molecular detection of *bla*NDM-1 gene



*-P is Positive; N is Negative; L is Ladder

Figure 26 Results of genotyping – molecular detection of *bla*OXA-51 gene



*-P is Positive; N is Negative; L is Ladder

5.18 COMPARISON BETWEEN PHENOTYPIC AND GENOTYPIC TEST RESULTS OF *CRE*:

Twenty one out of 25 (84%) tested CRE isolates were found positive for atleast one of the target genes. Out of the 25 isolates that were tested for *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-51}}$, 20(80%) were found positive for *bla*$_{\text{NDM-1}}$ gene and 8(40%) were positive for *bla*$_{\text{OXA-51}}$ gene. 7(28%) isolates were positive for both the genes. 13(52%) were found positive for *bla*$_{\text{NDM-1}}$ alone and was *bla*$_{\text{OXA-51}}$ negative. 1(4%) was found positive for *bla*$_{\text{OXA-51}}$ alone and was *bla*$_{\text{NDM-1}}$ negative. Out of the 20 *bla*$_{\text{NDM-1}}$ positive isolates, 11 (55%) were *Klebsiella spp.,* 7(35%) were *E.coli* and 2(10%) were *Proteus spp.,* 11 out of 16(68.75%) *Klebsiella spp., 7* out of 7 *E.coli* (100%) and 2 out of 2 *Proteus spp.,* isolates were found positive for *bla*$_{\text{NDM-1}}$. The details have been furnished in table 19.

Table 19 Comparative results – Phenotyping and genotyping

S.NO	CRE ISOLATE	KPC	MHT	EDS TEST	CDT	AMP C TEST	*bla*NDM-1	*bla*OXA-51
1	*Klebsiella spp.*	+	+	-	-	+	N	P
2	*Klebsiella spp.*	+	+	+	+	-	P	P
3	*Klebsiella spp.*	+	+	+	+	-	P	P
4	*Klebsiella spp.*	+	+	+	+	-	P	N
5	*Klebsiella spp.*	+	+	+	+	-	P	P
6	*Klebsiella spp.*	+	-	-	-	-	N	N
7	*Klebsiella spp.*	+	-	-	-	-	N	N
8	*Klebsiella spp.*	+	-	+	+	+	P	N
9	*Klebsiella spp.*	+	-	+	+	+	P	N
10	*Klebsiella spp.*	+	-	+	+	-	P	N
11	*Klebsiella spp.*	+	-	+	+	+	P	N
12	*Klebsiella spp.*	-	-	+	+	+	P	N
13	*Klebsiella spp.*	+	-	+	+	+	P	P
14	*E.coli*	+	+	+	+	-	P	N
15	*E.coli*	+	+	+	+	-	P	N
16	*E.coli*	+	+	+	+	-	P	P
17	*E.coli*	+	+	+	+	-	P	N
18	*E.coli*	+	+	+	+	-	P	N
19	*E.coli*	+	+	+	+	-	P	N
20	*Proteus spp*	+	+	+	+	-	P	N
21	*Proteus spp*	+	+	+	+	+	P	P
22	*Proteus spp*	+	+	+	+	-	P	P
23	*E.coli*	-	-	-	-	+	N	N
24	*Klebsiella spp.*	+	+	-	-	+	N	N
25	*Klebsiella spp.*	-	+	+	+	-	P	N

6. DISCUSSION

Drug resistant *Enterobacteriaceae* isolates have been found to cause many hospital and community acquired infections, *Carbapenemase producing Enterobacteriaceae* (CRE) being the most important and highly resistant amongst them all, causing high morbidity and mortality, making it imperative to conduct this study.

In this study, *Carbapenemase producing Enterobacteriaceae* were identified from heterogeneous clinical samples, their prevalence in various samples was studied, their distribution with respect to parameters like age, gender and clinical samples were analysed and their sensitivity patterns were studied. In addition, the different types of Carbapenemases were identified according to the phenotypic and genotypic methods.

6.1 SAMPLES RECEIVED IN OUR LABORATORY:

Urine (39.89%) was the most frequent sample received in our study, followed by pus (28.63%), sputum (17.87%), blood (8.4%) and body fluids (5.21%). The reason for this could be Urinary tract infection (UTI) being the most common hospital acquired infection accounting for more than forty percent.[49]

6.2 TOTAL NUMBER OF GRAM NEGATIVE BACILLI AND *ENTEROBACTERIACEAE* ISOLATED DURING THE PERIOD OF STUDY:

The number of culture positives in total from all clinical samples during the study period was 4423(66.5%) which included 347(5.3%) gram positive cocci and 2038 (30.6%) gram negative bacilli**.** The remaining 4266 (64.1%) included commensals, contaminats and samples where no growth were observed. This is in

concordance with the study by Sankarankutty et al where Gram negative isolates constituted 70.86% of culture positives.[50]

Out of 2038 gram negative bacilli, 1421(69.72%) were *Enterobacteriaceae* isolates and the remaining 617(30.27%) were non-fermenters. This finding is in concordance with the study by Lockhart et al where *Enterobacteriaceae* constituted 60% of the gram negative bacilli. It is also in agreement with the study by Sankarankutty et al where *Enterobacteriaceae* constituted 84.8% of the Gram negative isolates[50].

In our laboratory, the commonest *Enterobacteriaceae* isolated is *E.coli* (44.6%). The second most common is *Klebsiella spp., (*39.2%), followed by *Proteus spp.,* (9.9%), *Citrobacter spp.,* (3.4%), *Providencia spp.,* (1.4%), *Serratia spp.,* (1.1%) and *Salmonella spp.,* (0.4%). This finding is concordant with the results a study by Sankarankutty et al where *E.coli* (55.3%) was the predominant member of *Enterobacteriaceae* that was isolated, followed by *Klebsiella spp.,* (17.64%), *Proteus spp.,* (4.7%) *Citrobacter spp.,*(3.92%), *Enterobacter spp.,* (2.74%) and *Providencia* spp., (1.96%) in that order[50].

Pus (41.4%) is the sample from which most of the *Enterobacteriaceae* isolates were obtained in this study, followed by urine (33.9%), sputum (20.1%), blood (2.4%) and body fluids (2.2%). In a study by Sankarankutty et al, most of the *Enterobacteriaceae* were the isolates from Urine (72.60%), pus (18.26%), sputum (6.96%) and blood (1.3%) in that order[50]. Pus was the predominant sample harbouring *Enterobacteriaceae* in this study. This might be due to the reception of more number of pus samples from surgical ward patients and post operative patients in our setting.

6.3 SAMPLE WISE PREVALENCE OF ENTEROBACTERIACEAE ISOLATES:

In pus samples, *Klebsiella spp., (41.2%)* was the predominant isolate, followed by *E.coli (36.2%), Proteus spp.,(15.8%), Citrobacter spp.,(3.05%), Serratia(2.6%)* and *Providencia(1.2%).* This is in concordance with the study by Sunilkumar et al where *Klebsiella spp.,*(50%) was the predominant isolate from pus samples[51]. In a study by Soumya et al *E.coli*(50%) was the predominant isolate in pus samples, followed by *Proteus spp.,*(23.8%), *Klebsiella spp.,*(19.05%), *Citrobacter spp.,*(4.76%) and *Providencia*(2.4%)[50]. This discordance may be due to a relatively higher prevalence of *Klebsiella spp.,* in our hospital, especially in post operative patients from whom most of the pus samples were received.

In sputum samples, *Klebsiella spp., (67.1%)* was the predominant isolate, followed by *E.coli (16.8%), Proteus spp., (9.8%)* and *Citrobacter spp., (6.3%),* which is in correlation with the study by Vesna where *Klebsiella spp.,* was the predominant isolate from sputum samples in patients with infectious diseases of the respiratory tract[52].

In urine samples, *E.coli (73.4%)* was the predominant isolate, followed by *Klebsiella spp.,(17.4%), Proteus spp.,(4.2%), Citrobacter spp.,(2.5%)* and *Providencia(2.5%).* This is in concordance with many studies, an article by Sobia et al being one of them where *E.coli* was the predominant pathogen isolated from urine samples and is the cause for 85% of urinary tract infections[53].

In blood, *Klebsiella spp., (54.5%)* was the predominant isolate, followed by *E.coli(27.3%)* and *Salmonella(18.2%)*in our study, which is in concordance with the

results of a study by Jose et al, where *Klebsiella spp.,(26.3%)* was the predominant isolate from blood in patients with blood stream infections, followed by *E.coli* (21 %).[54]

In body fluids, *Klebsiella spp., (65.6%)* was the most commonly isolated, followed by *E.coli(31.2%)* and *Citrobacter spp.,(3.2%).* Most of the isolates were from Broncho alveolar lavage (BAL) fluid (50%) and *Klebsiella spp.,* (12/16 constituting 75% of Enterobaceriaceae isolates from BAL fluid) was the predominant Enterobacteriaceae isolate from BAL fluid in our study, followed by *E.coli* (4/16 constituting 25%)

E.coli was the predominant isolate from peritoneal fluid in our study (3 out of 6, 50%), followed by *Klebsiella spp.,* (2 out of 6) and *Citrobacter spp.,* (1 out of 6). According to an article by Thiago et al published in 2011, *E.coli* (70%) was the commonest microbe to be isolated from cases of bacterial peritonitis, followed by *Klebsiella spp.,* which is in concordance with our study[55].

*Klebsiella spp.,*was the only member of *Enterobacteriaceae* isolated from synovial fluid. The most frequently isolated member of *Enterobacteriaceae* from synovial fluid was *E.coli* according to a study by Mark et al[56].

Both *Klebsiella spp., and E.coli* were isolated from pleural fluids – 1 each. This is in concordance with the results of a study by Moon et al where *Klebsiella spp.,* was the predominant isolate from pleural fluids.[57]

6.4 PREVALENCE OF CRE:

Out of all the 1421 *Enterobacteriaceae* isolates, 56(3.94%) were found to be resistant to Meropenem by disc diffusion assay according to 2016 CLSI guidelines. So in our setting, the prevalence of *Carbapenem resistant Enterobacteriaceae (CRE)* according to our study was 3.94%. Yanling et al. in an Asian study have detected that the prevalence of CRE was 0.6% in 2001 and 1.3% in 2012[58]. Comparison of our study results with that of Yangling et al shows that Carbapenem resistance has been gradually rising since last decade. The overall incidence rate of CRE was 2.93% according to a study by Alice et al in 2015[59]. Datta P et al found the prevalence of CRE in their study to be 7.87%.[60] The prevalence of CRE according to the study by Nair P K et al was 12.26%[61].The results of all these studies clearly indicate an increasing incidence of Carbapenem resistance all over, warranting immediate action to stop emergence of further resistance.

6.5 AGEWISE DISTRIBUTION OF CRE:

In our study, patients above 60 yrs of age are found to be predominantly affected, constituting 37.5%, which is similar to the results of Meenakumari et al where the majority of patients infected by CRE were above 60 years of age. This finding also correlates with a study by Brennan et al where most of the patients infected with *CRE* were above 60 years of age[62].The high susceptibility of this age group may be due to the high prevalence of co-morbid conditions like Diabetes, Chronic Kidney disease (CKD), cancer and other immunocompromised conditions in this group[63].

6.6 GENDERWISE DISTRIBUTION OF CRE:

In this study there was an increased prevalence of CRE in males (62.5%) compared to females(37.5%).This finding is concordant with the findings of Gabriela et al., where the prevalence of *Carbapenemase producing Enterobacteriaceae* was higher in males (72.5%) compared to females (27.5%). This finding is also in concordance with a study by Kalidas et al., where the prevalence in males was 55% and it was 45% in females. 51% of the CRE infected patients were males according to a study by Brenner et al[62]. This higher male preponderance may be due to the associated non modifiable risk factors in males like their male gender itself, age, increased incidence of diabetes, Hypertension and CKD[63]. Though females are susceptible to these factors, they are protected to a greater extent by their hormonal factors until they attain menopause at an average age of 51 years, after which both the genders are equally susceptible to the risk factors[64].

40% of Urinary tract infections in our study group have occurred in postmenopausal women. Increased incidence of Urinary tract infection in females of postmenopausal age group can largely be attributed to the alkaline pH of the genitourinary epithelium due to depletion of lactobacillus, which had protected them in their premenopausal age[65].

6.7 PREVALENCE OF CARBAPENEM RESISTANCE IN EACH MEMBER OF ENTEROBACTERIACEAE:

Twenty one out of 634(3.31%) *E.coli* isolates, 25 of 557(4.49%) *Klebsiella spp.*, 8(5.67%) of 141 *Proteus spp.*, and 2(4.08%) out of 49 *Citrobacter spp.*, were found to be resistant to Meropenem and so the prevalence of Carbapenem resistant

Proteus spp., E.coli, Klebsiella spp., and Citrobacter spp., according to this study are 5.67%, 3.31%, 4.49%, and 4.08% respectively. The prevalence of Carbapenem resistant *E.coli, Klebsiella spp., and Enterobacter spp.,* were 7.3%, 4.1% and 0.9% respectively according to a study by Nair et al which correlates with our study results[61].

6.8 SAMPLE WISE DISTRIBUTION OF CRE:

Forty out of 589 (6.79%) *Enterobacteriaceae* from pus samples, 11 out of 285(3.86%) from sputum samples and 5 out of 482(1.04%) from urine samples are the prevalence of CRE in each sample according to our study. In a study by Nair et al, 46% of CRE were from urine samples, 16% from pus and 7% from sputum samples[61]. The higher prevalence of CRE from pus samples in our study shows that the surgical and post-operative wards of our healthcare setting are lagging behind in infection control practices, as most of the pus isolates in our study were received from surgical and post-operative wards.

Out of the 56 *CRE* isolated in our study, most of them were from pus samples which included drainage tube (71.42%), followed by sputum (19.64%) and urine (8.73%) samples. There were no *CRE* isolates from blood and body fluids. This finding is in concordance with a study by Saveetha et al where most (52%) of the isolates were from pus samples[66]. In a study by Pratita et al, pus (24%) was the second common sample from which *CRE* were isolated next only to urine[67].

6.9 ORGANISM WISE DISTRIBUTION OF CRE:

Out of the 56 CRE isolated in this study, 25(44.64%) were *Klebsiella spp.*, *spp.*, 21(37.5%) were *E.coli*, 8(14.29%) were *Proteus spp.*, and 2(3.6%) were *Citrobacter spp.*,

In this study, *Klebsiella spp.*, was the predominant Carbapenemase producer, followed by *E.coli*, *Proteus spp.*, and *Citrobacter spp.*, which are concordant with a study by Rosmari et al where the most common member of *Enterobacteriaceae* producing Carbapenemase was *Klebsiella spp.*, and the second most common was *E.coli, followed* by *Proteus spp.*, and *Citrobacter spp.*, in that order[68]. It is also similar to the finding of a study conducted by Chuang et al., where *Klebsiella spp.*, (58.1%) was the predominant isolate followed by *Enterobacter* (26.5%) and *E.coli* (9.4%).

6.10 *CRE* PREVALENT IN INDIVIDUAL SAMPLES:

In pus samples *Klebsiella spp.*, (50%) was the predominant isolate, followed by *E.coli* (32%), *Proteus spp.*, (13%) and *Citrobacter spp.*, (5%) This finding is in concordance with a study by Vijeta et al where *Klebsiella spp.*, (28%) was the CRE that was predominant in pus samples, followed by *Proteus spp.*, (5%), *Ecoli* (3%) and *Citrobacter spp.*, (2%). In a study by Nair P K et al, *Klebsiella spp.*, was the predominant CRE isolated from pus samples[61], which is also in concordance with our study.

E.coli was the predominant CRE in sputum samples according to this study (54.64%), which was followed by *Klebsiella spp.*, (27.36%) and *Proteus spp.*, (18%). In a study by P K Nair et al, *Klebsiella spp.*,was the predominant isolate from sputum samples[61]. *E.coli* was isolated from sputum in a study by Yang et al.

In urine samples *E.coli* and *Klebsiella spp.,* (40% each) were equally predominant followed by *Proteus spp., (20%)* in this study. According to the study by Manikandan et al., *E.coli* was the predominant *CRE* isolated from urine samples.[70] *E.coli* (67.6%), was found to be the predominant *CRE* causing urinary tract infection followed by *Klebsiella spp.*,(8.8%) and *Proteus spp.*,(5.2%) in a study by Enrico et al[71]. Also in a study conducted by Vasoo et al., Carbapenem resistant *E.coli* was the predominant isolate (72%) from urine samples followed by *Klebsiella spp.,* (15%) and *Proteus spp.,* (7%).The results of these studies are concordant with the results of our study.

The prevalence of Urinary tract infection was found to be more in females (3/5 – 60%) compared to males (2/5 – 40%) in our study. This is also in concordance with the study by Enrico et al where female to male urinary tract infection ratio was found to be 3.8[71]. According to a study conducted by Setegn et al., CRE causing Urinary tract infections were more common in females than in males[72]. The higher incidence of UTI in females may be attributed to short urethra in females, which favours easy spread of infection[73].

Three out of 5(60%) urinary *CRE* isolates were from patients above 50 years of age in our study, 2 out of 3 from patients above 60. This finding is also concordant with the results of study by Enrico et al where patients above 60 harboured most of the *CRE* isolates,[71]which are indicative of the vulnerability of the above 50 (especially postmenopausal females) age group to urinary tract infections due to multiple factors.[73]

6.11 PREVALENCE OF CRE IN INPATIENTS VS OUTPATIENTS:

In this study, most of the *Carbapenemase producing Enterobacteriaceae* isolates were isolated from inpatients (89.28%) and very less isolates were from outpatients (10.72%). This finding is in concordance with the study by Chaung et al where 70.5% of the CRE infections were acquired from hospital settings and only 29.5% from community settings.[69]This result is also in concordance with the study by Mamta et al where more than 90% of the isolates were from inpatients and less than 10% from outpatients[67]. These findings are indicative of most of the drug resistant infections being hospital acquired rather than community acquired and prolonged hospitalisation is a strong risk factor for acquision of these resistant bugs, showing the necessity for isolation of patients infected with drug resistant organisms, avoidance of irrational use of antibiotics on inpatients and strict adoption of hand hygiene measures like handwashing/ application of hand rub by the medical and paramedical personnels before handling each patient.

6.12 PREVALENCE OF *CRE* IN RELATION TO WARDS IN HOSPITAL:

In our study, Surgical ward is the ward where most CRE has been isolated (26 out of 56 making 46.43%), followed by Medicine wards (10.71%), Chest and TB ward and OG ward (8.93% each), Ortho ward (7.14%), ICU and casualty (5.36% each), and burns and dermatology ward (3.57%) each.

This distribution is in concordance with the study results of Gabriella et al., where majority of the CRE were from patients in Surgical wards constituting thirty percent and Intensive Care Units. This coincides with the results of Alves study, where the greatest isolation of about thirty two percent occurred in the surgical clinic

and nearly the same number from internal medicine specialties. These results are also in concordance with a study by P k nair et al where most of the CRE isolates were from surgical ward, followed by ICU[61].

Klebsiella spp., is the predominant CRE from surgical wards (13/26), followed by *E.coli*(7/26) in our study. This result is in concordance with a study by Fortunata et al where *Klebsiella spp.,* is the predominant CRE isolate from surgical wards.[74] This finding is also in concordance with a study by PK Nair et al where *Klebsiella spp.,* is the predominant isolate from surgical wards[66]. *E.coli* is the predominant isolate from medicine ward, especially from patients suffering from infections of the urinary tract in our study. This result is also concordant with the results of study by PK Nair et al[66]. From ICUs, *E.coli,* species of *Klebsiella and Proteus* were equally isolated in our study, which is similar to the study by PK Nair et al[66].

6.13COMORBID CONDITIONS & RISK FACTORS FOR CRE INFECTIONS:

CRE from pus have been isolated from various cases like diabetic ulcer, Ileostomy wounds, Bed sores, surgical wounds, CKD patients, infected vulval cyst, cervicitis and pelvic/ gluteal abscesses. Diabetic foot is the most common clinical condition associated with CRE infection in this study. Type 2 diabetes mellitus [24/56(43%)] is the most common co morbid condition associated with CRE infected patients in this study which is concordant with the results of Alice et al. where Diabetes mellitus is the most common co-morbid condition (44.3%) associated with CRE infected patients[59]. Diabetes mellitus is as such an immunocompromised state which makes the patient easily susceptible to infections with drug resistant organisms.

CKD is also another co morbid condition leading to CRE infections according to this study. This is in concordance with the study by Garbarti et al where CKD was the predominant co-morbid condition[75] (48.3%). Both diabetes mellitus and chronic kidney disease are immunocompromised states, which show that immunocompromised patients are at increased risk of acquiring CRE infection. This finding also correlates with a study by Ling et al where diabetes mellitus, cardiovascular disease, renal disease and cancer were the most common co-morbid conditions associated with CRE infections. Recent surgery (post operative state) is another common risk factor in this study which also correlates with their study.[76]

Hospitalisation, especially prolonged hospitalisation for more than a week has been identified as a risk factor in this study, as it was found that all the in-patients harbouring CRE isolates in their clinical samples had been hospitalised for more than a week. Prolonged hospitalisation is an independent risk factor for infections with drug resistant organism[76].

Prolonged antibiotic usage is another independent risk factor in our study, for the patient being infected with drug resistant CRE, as about 90% of the CRE harbouring patients in this study were on antibiotic treatment for more than week while in hospital. So irrational or overuse of antibiotics have to be avoided to prevent spread of drug resistant organisms[76].

E.coli is the most common isolate from patients who have undergone abdominal surgeries like ileostomy and laparotomy [4/6 (66.6%)] in this study. *E.coli* and

Proteus spp., are the CRE isolated from two burns patients included in this study. One Carbapenem resistant *Citrobacter spp.,* was isolated from Ileostomy wound.

18/56(32.14%) of the CRE isolates were from postoperative cases in this study, *Klebsiella spp., (50%)* being the predominant isolate. This finding is concordant with that of an Indian study by Sunilkumar et al where postoperative patients were very commonly infected with Carbapenemase producing *Klebsiella spp.,*[51]

6.14 DRUG SENSITIVITY PATTERN OF *CRE* IN OUR STUDY:

In this study, most of the isolates resistant to Meropenem were resistant to all the routinely used drugs for AST by Kirby Bauer disc diffusion testing (AMP, COT, CIP, CTR, CFS, PIT and IPM). However, a few isolates were sensitive to some of the drugs. 2 Carbapenem resistant *E.coli* and 4 Carbapenem resistant *Klebsiella spp.,* isolates were sensitive to Gentamycin (overall sensitivity to G was 16.1%), as determined the routine Kirby Bauer disc diffusion testing according to current CLSI guidelines. 6 *E.coli,* 6 *Klebsiella spp.,*, 1 *Citrobacter spp.,* and 2 *Proteus spp.,* were sensitive to Amikacin (overall sensitivity to AK was 26.98%). All other isolates were resistant to all other drugs that were tested for. The findings are similar to the results of a study carried out by Betina et al., where some of the isolates resistant to Imipenem showed sensitivity to Aminoglycosides like Gentamycin and Amikacin.

All the Meropenem resistant isolates were found to be susceptible to Tigecycline and Colistin (100% each) as tested by the Kirby Bauer disc diffusion assay. This finding is also in concordance with the results of Betina and Radha Srinivasan et al. where all the Carbapenem resistant isolates were sensitive to Colistin and Tigecycline[77]. These results suggest that Colistin and Tigecycline can be used for

the treatment of CRE infections. Though some studies have reported emerging resistance of gram negative bacilli to these newer drugs, extensive resistance has not yet occurred, and it's time to protect these drugs from the superbugs through proper usage of these drugs only when they are absolutely indicated, that too as a part of combination therapy[45].

6.15 PHENOTYPIC TESTS:

Among various methods of Carbapenemase and AmpC β-lactamase detection in *Enterobacteriaceae*, phenotypic methods are widely employed for routine testing because of their technical simplicity and cost-effectiveness. However, results of these phenotypic methods are essentially affected by several environmental and technical factors, such as, temperature, incubation period, quality and thickness of media, inoculums characters, distance between the antibiotic disks and subjective errors[78]. Growth on KPC chrome ager, Modified Hodge test, Ethylene diamine tetra acetic acid disc synergy test (EDS) and Combined disc test(CDT) for Carbapenemase detection & AmpC disk test for AmpC β-lactamases have been used in this study, as these tests were easy to perform , and they were also found to have considerable sensitivity and specificity as detected by previous studies.

6.15.1 GROWTH ON KPC CHROM AGAR:

When the isolates resistant to Meropenem were tested by KPC chrome agar, 52/56 isolates (92.86%) grew on chrome agar with specific colours indicating Carbapenemase production. The isolates included 19/21 *E.coli* (90.48%), 23/25 *Klebsiella spp.,* (92%), 8/8 *Proteus spp.,* (100%) and 2/2 *Citrobacter spp.,* The remaining four CRE isolates that did not grow on KPC chrome agar might have been

resistant to Meropenem due to mechanisms other than Carbapenemase production, such as porin loss, efflux of antibiotics or hyperproduction of Amp C enzymes[31].KPC CHROM agar has a high sensitivity of 100% and specificity of 98.4% for detecting CRE[38]. In a study by Akshaya rao et al, KPC chrome agar was found to detect Carbapenemase production in 96.6% of CRE isolates[79]. KPC, IMP, VIM, NDM, and OXA-48-producing CRE can be detected by KPC chrome agar[38]. A study by Nordmann et al has found that KPC chrome agar had a relatively higher sensitivity for detecting class A Carbapenemases (67%) and lower sensitivity for other classes - 56% for class B and 14% for class D[41].

6.15.2 MODIFIED HODGE TEST:

All the 56 isolates were tested by MHT. 38(67.86%) were MHT positive and the remaining 18 were negative (12.14%). This result is in concordance with the study by Priyadharshini et al, where 82% of the CRE isolates were MHT positive[80]. It is also in concordance with the study by Pratita et al where 60% of the CRE isolates were MHT positive[67].

MHT positives in our study consisted of 17 (45%) *Klebsiella spp., spp.,* 15 (39%) *E.coli,* 6 (16%) *Proteus spp.,* and no *Citrobacter spp.* In a study by Amjad et al., 38% of *E.coli, 17% of Klebsiella spp., pneumoniae* (17%), *2% Citrobacter spp., diversus* (2%) were MHT positive[81]. According to a study by Pratita et al, *E.coli* was the predominant organism positive for MHT[67]. MHT is the phenotypic confirmatory test for Carbapenemases according to CLSI. It is most sensitive for detection of KPC Carbapenemases.[37] It has varying sensitivity for other types of Carbapenemases. It is not done as a routine, but done for epidemiological purposes[37]. It can also give false

positives with ESBL and Amp C producers. PCR is the confirmatory test. With all these, only 70% positivity in our study may be due to the other CRE isolates producing non class A Carbapenemases, which can be confirmed by genotyping.

6.15.3 EDTA DISC SYNERGY TEST:

EDS test was performed on all 56 isolates, out of which 44(78.57%) were found to be positive i.e. MBL producers and 12 (21.43%) were negative i.e. the prevalence of MBL producing CRE according to our study was 78.57%. This finding is concordant with the study by Rajkumari et al where the prevalence of MBL producing CRE was 90%[82].

Among the 44 isolates that tested EDS test positive 15(34.09%) were *Klebsiella spp.,* 19(47.5%) were *E.coli,* 8(20%) were *Proteus spp.,* and 2(5%) were *Citrobacter spp., spp.,* Thus *E.coli* was the predominant MBL producer according to our study. This finding is concordant with the results of the study by M Choudary et al ,where *E.coli* was the predominant MBL producing CRE (37.5%) followed by *K.pneumoniae* (27.8%). NDM is the predominant MBL found in India. In a study by Diene et al, *Klebsiella spp.,* was the predominant NDM producer followed by *E.coli*[83]. The result is also concordant with a study by Rajkumari et al where *E.coli* was the predominant MBL producer followed by *Klebsiella spp.,*[82]

The sensitivity and specificity of EDS test to detect MBL producers are 95% and 92% respectively according to a study by Young et al. A study by Lee et al. has proved 100% sensitivity and specificity.[41]Many bacterial isolates that produce Metallobetalactamases are not detected by the MHT, but can be detected by the sensitive EDTA disk synergy test.[41]

6.15.4 COMBINED DISC TEST (CDT):

Out of the 56 isolates tested, 44(78.57%) were positive for MBL production as detected by the CDT (Combined disc test) in our study. The ability of the test to detect MBL producers is similar to that of EDTA disc synergy test. Anyway PCR is confirmatory. Behera et al. reported equal efficacy of both combined disk test and E test.[84]Among the 44 isolates that tested CDT positive 15(34.09%) were *Klebsiella spp.*, *spp.*, 19(47.5%) were *E.coli*, 8(20%) were *Proteus spp.*, and 2(5%) were *Citrobacter spp.*, which is similar to that detected by EDS test in our study.

6.15.5 AMP C DISC TEST:

Out of 56 Carbapenem resistant isolates tested, 26(46.43%) were identified as Amp C producers by Amp C disc test, which included 14(53.85%) *Klebsiella spp.*, *spp.*, 6(23.07) *E.coli*, 4(15.38%) *Proteus spp.*, *spp.*, and 2(3.57%) *Citrobacter spp.*, *spp.*, A study by Jennifer et al., has reported that the sensitivity and specificity of Amp C disc test in detection of AmpC enzymes that are encoded on plasmids are 98% and 100% respectively[85]. The mechanism of Carbapenem resistance in Amp C producers is that Amp C production causes low influx and high efflux of the antibiotic due to outer membrane porin loss and efflux pump activation respectively[85].

6.16 CUMULATIVE RESULTS OF THE PHENOTYPIC TESTS OF THE CRE IN THIS STUDY:

According to the phenotypic test results, *E.coli* is positive for all phenotypic tests indicating that it is a producer of all types of Carbapenemases (KPC, MBL, OXA) and also Amp C, which is concordant with a study by Gisele et al where E.coli was found to produce all types of Carbapenemases[86]. The proportion of *E.coli*

producing Amp C is lower (28.57%) compared to other organisms. *Klebsiella spp.,* and *Proteus spp.,* are producers of all classes of Carbapenemases and are also Amp C producers. All *Proteus spp.,* and *Citrobacter spp.,* isolates were found to produce one or the other type of Carbapenemase by their growth on KPC chrome agar. Two *Klebsiella* isolates were found to be negative for all tests except Amp C disc test which implies that high grade Amp C production can result in Carbapenem resistance, even in the absence of Carbapenemase production. The proportion of MBL production was more (100%) in *Proteus spp.,* and *Citrobacter spp.,* when compared to *Klebsiella* and *Escherichia* isolates. The proportion of Ambler class C production was highest in *Citrobacter spp.,* (2/2) followed by *Klebsiella spp.,, Proteus spp., and E.coli* in that order. *Klebsiella pneumoniae* is the most common Carbapenemase producer altogether (25/56 isolates), followed by *E.coli* (21/56), *Proteus spp.,* (8/56) and *Citrobacter spp.,* (2/56). This may be attributed to the rapid plasmid mediated transfer of resistant genes in *Klebsiella*.[10]

Growth on KPC chrome agar and Modified Hodge test have picked up 92.86% and 67.86% of the CRE isolates respectively as Carbapenemase producers. EDS test and combined disc test, both have picked the same 78.57% of the isolates as Carbapenemase producers. These findings show that Modified Hodge test is the least sensitive and growth on CHROME agar KPC is the most sensitive for the detection of Carbapenemases. EDS test and CDT both have the same sensitivity as detected by our study. Both these tests have a higher sensitivity than Modified Hodge test and a lower sensitivity than CHROME agar KPC for the detection of Carbapenemases. This finding is concordant with the study results of Aparna et al [87].

6.17 PREVALENCE OF THE TARGET GENES (*bla*$_{NDM-1}$ and *bla*$_{OXA-51}$) IN THE CRE ISOLATES IN THIS STUDY:

The target genes in this study were *bla*$_{NDM-1}$ and *bla*$_{OXA-51}$. 21 out of 25 (84%) tested CRE isolates were found positive for atleast one of the target genes. Twenty out of 25 (80%) were found positive for *bla*$_{NDM-1}$ gene and 8(40%) were positive for *bla*$_{OXA-51}$ gene. 7(28%) for both the genes, i.e. 28% were co-producers of Metallobetalactamase and Oxacillinase. 13(52%) were found positive for *bla*$_{NDM-1}$ alone and *bla*$_{OXA-51}$ negative. 1(4%) was found positive for *bla*$_{OXA-51}$ alone and was *bla*$_{NDM-1}$ negative. In a study published in 2016 by Mubin et al, the target genes were detected in 88% of the CRE isolates, the prevalence of *bla*$_{NDM}$ were 62.5%, the prevalence of *bla*$_{OXA}$ were 12% and the prevalence of isolates harbouring both *bla*$_{NDM}$ and *bla*$_{OXA}$ were 31.8%. In a study by Jyothi et al conducted in Bangalore and published in 2014, the prevalence of NDM-1 producers was 93.24% (69 of the total of 74 MDR isolates) [88]. The result of our study is almost similar to the results of these two studies with only slight variations.

11 out of 16(68.75%) *Klebsiella spp.,* 7 out of 7 *E.coli* (100%) and 2 out of 2 *Proteus spp.,* that were tested were found positive for *bla*$_{NDM-1}$. The result of this study is in concordance with a study by Chandran et al in 2012, conducted in Bangalore, where 75% of the *Klebsiella spp.,*isolates were NDM positive.[89] It is also in concordance with an Indian study by Bora et al published in 2013, where all the 14 *E.coli* isolates (100%) tested for *bla*$_{NDM}$ were found to be positive.[90] Majority of the NDM producing isolates were *Klebsiella spp.,* and *E.coli,* which is also in concordance with the study by Bora et al.[90]

6.18 COMPARISON OF THE PHENOTYPIC TEST RESULTS WITH GENOTYPIC TEST RESULTS:

All the isolates that were found to be positive for *bla*$_{NDM-1}$ by genotyping were found positive for EDS test and CDT. This shows that EDS and CDT are 100% sensitive for the detection of Metallobetalactamses. And all the isolates that tested negative by the phenotypic EDS test and CDT also tested negative for *bla*$_{NDM-1}$ gene by genotyping. This shows that EDS and CDT are 100% specific. A study by Lee et al. has also proved 100% sensitivity and specificity for EDS test. In a study by Jyothi et al, CDT detected more than 97% of the NDM-1 producers[91]. All these results prove EDS test and CDT to be the two most reliable phenotypic tests for detection of Metallobetalactamase producers.

All the *CRE* isolates that were found to harbour NDM-1 or OXA-51 were found positive for growth on KPC chrome agar. So this study shows that KPC CHROME agar is highly efficient in detecting NDM-1 and OXA-51 producers. A study by Samra et al in 2008 has proved KPC chrome agar to be 100 % sensitive and 98% specific for detection of Carbapenemases.[38]

All the 8 CRE isolates confirmed as *bla*$_{OXA-51}$ gene positive by genotyping in our study were found MHT positive, which shows that MHT has 100% sensitivity for picking up Oxacillinase producers. 14 out of 20(70%) *bla*$_{NDM-1}$ positive isolates confirmed by genotyping were found positive for MHT. Thus the sensitivity of MHT for detection of NDM according to this study is 70%. However, 7 out of 14 isolates were found to be positive for both *bla*$_{NDM-1}$ and bla OXA-51 genes, making the specificity of MHT for Metallobetalactamase detection doubtful, as the MHT

positivity in those 7 isolates might have been due to Oxacillinase production and may not be due to Metallobetalactamase alone. In a study by Jyothi et al conducted in Bangalore, published in 2014, 76.47% of Metallobetalactamase producers were identified by MHT[91], which is concordant with our results.

bla$_{NDM-1}$ gene is the one that encodes for NewDelhi Metallobetalactamase-1. It may either be located on chromosomes or on plasmids. If it is located on plasmid, the gene can carry many other resistance determining genes (upto 14) making the bacteria resistant to other group antibiotics in addition to being Carbapenem resistant.[91]This can result in the strain becoming multidrug resistant and even extremely drug resistant. The ability of these plasmid encoded resistant genes to be transferred to other bacteria adds fuel to the fire, making the condition even more worse because of rapid spread of resistance.[91]This explains why the Bacterial isolates resistant to Carbapenems in our study has been non susceptible to many other groups of drugs too.

NewDelhi metallobetalactamses are reported in larger numbers in India and across the world. [91] This study was therefore conducted to detect the prevalence of Carbapenemase producers among the members of *Enterobacteriaceae* harbouring *bla*$_{NDM-1}$ gene among the isolates that were Carbapenem resistant from a rural tertiary care centre in Trichy district in Tamilnadu. A high prevalence (80%) of NDM-1 among the Carbapenemase producing isolates have been identified here, and most of the isolates were also found to be multidrug resistant, i.e. resistant to more than three groups of antimicrobials.[92]

In our study, 40% (8 out of 25) of the isolates were positive for *bla*$_{\text{OXA-51}}$ gene. In a study by Niu et al conducted in China, published in 2015, *bla*$_{\text{OXA-51}}$ was identified in all the 93(100%) *Acinetobacter* isolates.[93] A study by Budak et al suggests *bla*$_{\text{OXA-51}}$ gene to be intrinsic to *Acinetobacter baumanii* and a patient with Ventilator associated pneumonia harbouring *bla*$_{\text{OXA-51}}$ gene in *Klebsiella pneumoniae* has been reported in their study. The reason for *bla*$_{\text{OXA-51}}$ gene in that *Klebsiella* isolate was suspected to be chromosomal or plasmid mediated transfer from *Acinetobacter*, as the patient was also found to be infected with *Acinetobacter*, for which he was treated with antibiotics. However, PCR and other conjugation experiments proved their suspicion false.[94]The reason behind the *Klebsiella pneumoniae* harbouring *bla*$_{\text{OXA-51}}$ remains obscure. In a study by Tolman et al in India in 2005, all the *Acinetobacter* isolates (100%) tested were found to harbour *bla*$_{\text{OXA-51}}$ gene. In a study by Leski et al, in Sierra Leone *bla*$_{\text{OXA51}}$ like gene was detected in 3/22(13.64%) *Klebsiella pneumoniae* isolates, 3/22(13.64%) *Enterobacter cloacae* isolates and 1 *E.coli* (4.5%) isolate suggesting that the gene is no longer confined to *Acinetobacter* alone[95]. Though there are a considerable number of studies on *bla*$_{\text{OXA-51}}$ in *Acinetobacter* in India, there are hardly any studies on *bla*$_{\text{OXA-51}}$ in *Enterobacteriaceae* in India, and that is the reason why molecular detection of *bla*$_{\text{OXA-51}}$ gene has been carried out in this study.

With all these results, it is obvious that Carbapenemase producing *Enterobacteriaceae* are prevalent in our setting, as shown by 13 NDM-1 Carbapenemase producers and one OXA-51 Carbapenemase producer and seven of the CRE isolates being producers of more than one type of Carbapenemases as detected by this study.

7. SUMMARY

This study entitled, **"PREVALENCE, PHENOTYPING AND MOLECULAR DETECTION OF *bla*$_{NDM-1}$ and *bla*$_{OXA-51}$ GENES IN CARBAPENEMASE PRODUCING STRAINS AMONG THE *CARBAPENEM RESISTANT ENTEROBACTERIACEAE*"** , was carried out in the department of Microbiology, Chennai medical college hospital and research centre, Trichy from October 2014 to December 2015.

- Over a period of fifteen months (October 2014 to December 2015), the laboratory received 6651 heterogenous clinical samples, from which 2038(30.6%) gram negative bacilli were isolated.
- Among the gram negative isolates, *Enterobacteriaceae* constituted 69.72% (N= 1421)
- The *Enterobacteriaceae* were isolated predominantly from pus samples (41.4%), followed by urine (33.9%), sputum (20.1%), blood (2.4%) and body fluids (2.2%).
- Of the *Enterobacteriaceae* isolates (1421), 56 were found to be Carbapenem resistant as identified by the disc diffusion assay and thus the prevalence of *CRE* by this method was 3.94%.
- Further analysis of these *CRE* in relation to clinical samples revealed that it was 71.43% (N=40) from pus, 19.64 % (N=11) from sputum and 8.73% (N=5) from urine. There were no Carbapenem resistant isolates from blood or other body fluids.

- The prevalence in relation to gender showed that *CRE* isolates were more in males (62.5%) and it was statistically significant.
- Males above 60 years of age were predominantly infected with *CRE* (37.5%) and diabetes mellitus was the most common co-morbid condition (42.86%) associated with *CRE* infections. Most of the *CRE* were isolated from patients admitted to surgical wards (71.43%).
- The antimicrobial sensitivity of the 56 *CRE* isolates to Gentamycin, Amikacin, Cefotaxime, Tigecycline and Colistin were in the order of 16.1, 26.8, 53.6, 100 and 100% respectively. These 56 strains were resistant to Ampicillin, Fluroquinolones, betalactam-betalactamase inhibitors.
- All the 56 CRE were subjected to various phenotypic tests which included growth on KPC CHROM agar, Modified Hodge test, EDTA disc synergy test, Combined disc test and AmpC disc test.
- Among the five phenotypic tests used, the highest number of CRE(52/56 – 92.86%) were detected by KPC CHROM agar followed by Combined disc and EDTA disc synergy tests in equal numbers (44/56 – 78.57%), and the least detected by Modified Hodge test (38/56 – 67.86%). AmpC disc test detected (30/56-53.57%) of CRE isolates as AmpC producers.
- Out of 56 CRE, 25 belonging to odd numbers were subjected to genotyping of *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-51}}$ genes. The analysis revealed that 7 were positive for both, 13 others for bla$_{\text{NDM-1}}$ and one other for bla$_{\text{OXA-51}}$ alone.
- Comparative analysis of phenotyping and genotyping carried out for 25 CRE revealed that all the five phenotypic tests were positive only for one isolate

(*Proteus spp.*,) which carried both the genes. All the 21 isolates which were positive for bla$_{NDM-1}$ genes were also positive for growth on KPC CHROM agar, EDS test, CDT, showing that these three phenotypic tests are 100% efficient in detecting NDM. The 4 isolates bla$_{NDM-1}$ negative isolates were negative for EDS test and CDT, showing these two tests to have 100% specificity as well in detecting NDM.

- Comparative analysis of phenotyping and genotyping carried out for 25 CRE revealed that all the five phenotypic tests were positive only for one isolate (*Proteus spp*.,) which carried both the genes. A good correlation has been obtained between the phenotypic and genotypic tests suggesting the use of easier and cost effective phenotypic tests for routine detection of Carbapenemase producers, which can aid to a great extent in correct diagnosis and treatment patients infected with Carbapenemase producers. However, one has to rely on independent genotyping and subtyping to recognise molecular epidemiology towards the surveillance and prevention.

8. CONCLUSION

- In the present study, 1421 *Enterobacteriaceae* were isolated from 6651 heterogenous clinical samples.
- 56 of these 1421 were found to be resistant to Carbapenem by disc diffusion assay. Thus disc diffusion identified a prevalence of 3.94% as presumptive Carbapenemase producers.
- These CRE were isolated in more numbers from pus than from other biological samples. The CRE were predominant in males and were more in surgical wards.
- All these were resistant to commonly used antimicrobial agents like Ampicillin, Betalactam-betalactamase inhibitor combinations and Fluoroquinolones.
- Phenotypic tests performed on these 56 isolates with growth on KPC CHROM agar, MHT, EDS, CDT and Amp C disc tests revealed positivity in 92.86, 67.86, 78.6, 78.6 and 53.6% respectively indicating the ability of different phenotypic tests to detect different betalactamase enzymes depending on the biological properties of the isolates producing them.
- Twenty five of the 56 odd numbered CRE isolates were subjected to multiplex PCR for detection of bla_{NDM-1} and bla_{OXA-51} genes. 7 were found to carry both the genes, 13 with NDM gene alone and 1 with OXA gene alone.
- All the 20 bla_{NDM-1} gene positive isolates were found to test positive for growth on KPC CHROM agar, EDS and CDT which suggests that KPC CHROM agar, EDS and CDT are highly efficient in detecting NDM producers.

All the 5 isolates which were negative for EDS and CDT were also negative for bla$_{NDM-1}$ gene, suggesting that the EDS and CDT can rule out NDM non producers rightly. In the present study bla$_{OXA-51}$ was noticed among 8 of the 25 (40%) isolates. All these 8 isolates were found to be positive for MHT and growth on KPC, suggesting that MHT and KPC are highly efficient in detecting OXA producers. This makes one to consider the use of simple phenotypic methods in resource limited centres/ laboratories to aid in the diagnosis of the *Carbapenemase producing Enterobacteriaceae*.

- Inter relationship between the phenotypic and genotypic tests when analysed showed that the consistency between the two were considerably good. However, genotyping is the gold standard method for detecting the specific resistance determining genes which is required in subtyping in molecular epidemiology, surveillance system and recognition of transmission of resistant strains from one area to another.
- The prevalence of CRE in our setting is considerably low, probably due the rural population that is relatively naive to antibiotics. Even then, the presence of these superbugs itself strongly suggests the need to prevent their further spread, which can be done by appropriate use of antibiotics only when truly needed, appropriate hand hygiene measures, health education of the patients regarding strict compliance to prescribed antibiotic regimen and to avoid over the counter antibiotics, upto date vaccinations, proper handling of food and water, isolation of patients carrying drug resistant organisms. All these measures can prevent us from reaching the post antibiotic era where common

diseases can kill us once again. Carrying out researches/studies on drug resistant organisms, formulation of antibiotic policies for health institutions and Antibiotic stewardship programs can aid to a great extent in proper antibiotic usage and prevention of further spread of resistance, thereby paving a way for a healthy community.

STRENGTH OF THE STUDY:

- Five phenotypic tests on CRE have been compared and analysed.
- Clinical correlation has been done with respect to parameters like age, gender, inpatient/outpatient details and co-morbid conditions.
- Genotyping for the most common (*bla*NDM-1) and one uncommon gene (*bla*OXA-51) has been carried out.
- Genotyping results have been compared to the phenotyping results and the efficacy of each test has been analysed.

LIMITATIONS OF THE STUDY:

- Test sample size is low
- Genotyping has been done only for 25 out of the 56 CRE isolates.
- It is a single centre based study.

MASTER CHART:

Table showing Patient details, bacterial isolate and their drug sensitivity pattern

PUS ISOLATES:

S No	IP no	Age	Sex	Ward	Diagnosis	Comorbid condition	Organism	G	AMP	COT	CIP	CTX	CFS	CX	AK	AT	MRP	KPC	MHT	EDS	CDT	AMPC	blaNDM-1	BlaOXA-51	Clinical condition
1	164664	38	M	Surgery	Diabetic foot	T2 DM	*Klebsiella spp.*	R	R	R	R	R	R	R	S	R	R	+	+	-	-	+	N	P	Improved
2	163469	45	F	OG	TAH/BSO	-	*Klebsiella spp.*	S	R	R	R	R	R	R	R	R	R	+	-	-	-	-	ND	ND	Improved
3	161465	75	M	Surgery	Ileostomy	T2 DM	*E.coli*	R	R	R	R	R	R	R	R	R	R	+	-	+	+	-	ND	ND	Improved
4	164578	70	M	Surgery	Diabetic foot	T2 DM	*Klebsiella spp.*	R	R	R	R	R	R	S	R	R	R	+	+	+	+	-	P	P	Improved
5	164672	77	M	Derm	Bed sores	T2 DM	*Klebsiella spp.*	R	R	R	R	R	R	S	S	R	R	+	+	+	+	-	P	P	Expired
6	164664	38	M	Surgery	Diabetic foot	T2 DM	*Proteus spp*	R	R	R	R	R	R	S	S	R	R	+	+	+	+	+	ND	ND	Improved
7	162002	55	M	Ortho	AK amputatio	T2 DM	*Klebsiella spp.*	R	R	R	R	R	R	R	R	R	R	+	-	-	-	+	ND	ND	Improved

					n																			
8	164578	70	M	Surgery	Diabetic foot	T2 DM	*Proteus spp*	R	R	R	R	R	R	S	R	R	R	+	-	+	+	-	P	N	Improved
9	163859	50	M	ICU	CKD	T2 DM	*E.coli*	R	R	R	R	R	R	R	R	R	R	-	-	-	-	-	ND	ND	Improved
10	163007	40	F	OG	TAH	-	*E.coli*	R	R	R	R	R	R	S	R	R	R	+	+	+	+	-	P	N	Improved
11	163566	52	F	Surgery	Diabetic foot	T2 DM	*E.coli*	R	R	R	R	R	R	S	S	R	R	+	-	+	+	+	P	N	Improved
12	160549	38	M	Medicine	Pressure sore	T2 DM	*Klebsiella spp.*	R	R	R	R	R	R	R	R	R	R	+	+	-	-	+	ND	ND	Improved
13	164075	37	F	OG	Infected vulval cyst	-	*Klebsiella spp.*	R	R	R	R	R	R	S	R	R	R	+	+	+	+	-	P	N	Improved
14	161671	56	M	Ortho	ORIF, Locking plate	-	*E.coli*	S	R	R	R	R	R	R	R	R	R	+	+	+	+	+	ND	ND	Improved
15	162688	65	M	Surgery	Laparotomy	-	*E.coli*	R	R	R	R	R	R	R	R	R	R	+	+	+	+	-	ND	ND	Improved
16	161130	55	M	Surgery	Iliostomy	-	*Klebsiella spp.*	R	R	R	R	R	R	S	R	R	R	+	+	+	+	-	P	P	Improved
17	161130	55	M	Surgery	Iliostomy	-	*Citrobacter spp*	R	R	R	R	R	R	R	R	R	R	+	-	+	+	+	ND	ND	Improved

18	115447	55	M	Casualty	Diabetic foot	T2 DM	*Klebsiella spp.*	R	R	R	R	R	R	R	R	R	R	+	-	+	+	+	ND	ND	Improved
19	502954	54	M	Casualty	Diabetic foot	T2 DM	*E.coli*	R	R	R	R	R	R	S	S	R	R	+	+	+	+	-	P	P	Improved
20	1132061	55	F	OG	Chronic cervicitis	-	*E.coli*	R	R	R	R	R	R	S	S	R	R	+	-	+	+	+	P	N	Improved
21	162829	65	M	Surgery	Wound debridement	T2 DM	*E.coli*	S	R	R	R	R	R	S	R	R	R	+	+	+	+	-	P	N	Improved
22	162442	53	M	Surgery	Diabetic foot	T2 DM	*Proteus spp*	R	R	R	R	R	R	S	R	R	R	+	+	+	+	+	P	P	Improved
23	162230	54	M	Surgery	Diabetic foot	T2 DM	*Klebsiella spp.*	S	R	R	R	R	R	R	R	R	R	+	+	-	-	+	ND	ND	Improved
24	162623	65	M	Surgery	Diabetic foot	T2 DM	*Klebsiella spp.*	R	R	R	R	R	R	S	S	R	R	-	+	-	-	+	N	N	Improved
25	162458	48	F	Surgery	Diabetic foot	T2 DM	*Proteus spp*	R	R	R	R	R	R	R	R	R	R	+	+	+	+	+	ND	ND	Improved
26	162322	64	F	Surgery	Diabetic foot	T2 DM	*Klebsiella spp.*	R	R	R	R	R	R	S	S	R	R	-	-	-	-	+	N	N	Improved
27	162014	45	M	Surgery	Pyocele R-orchidectomy	-	*Citrobacter spp*	R	R	R	R	R	R	R	S	R	R	+	-	+	+	+	ND	ND	Improved

28	161232	61	M	Surgery	Diabetic foot	T2 DM	*Klebsiella spp.*	R	R	R	R	R	R	R	R	R	R	+	+	+	+	-	P	N	Improved
29	160404	75	M	Surgery	Diabetic foot	T2 DM	*Klebsiella spp.*	R	R	R	R	R	R	R	R	R	R	+	+	+	+	-	P	N	Improved
30	159763	68	F	Surgery	Abscess-both Thigh	T2 DM	*Klebsiella spp.*	S	R	R	R	R	R	R	R	R	R	+	+	+	+	+	ND	ND	Improved
31	160592	70	M	Surgery	R Gluteal abscess	T2 DM	*Klebsiella spp.*	R	R	R	R	R	R	R	R	R	R	+	+	-	-	+	ND	ND	Improved
32	1026388	35	F	OG	Pelvic abscess	-	*E.coli*	R	R	R	R	R	R	S	S	R	R	+	+	+	+	-	P	N	Improved
33	158141	57	M	Surgery	Diabetic foot	T2 DM	*Klebsiella spp.*	R	R	R	R	R	R	S	R	R	R	+	+	+	+	-	P	N	Improved
34	160502	25	M	Ortho	Wound debridement with EF	-	*Klebsiella spp.*	R	R	R	R	R	R	R	R	R	R	+	-	+	+	+	P	N	Improved
35	161431	40	F	Surgery	Diabetic foot	T2 DM	*Klebsiella spp.*	R	R	R	R	R	R	R	R	R	R	+	-	+	+	+	P	N	Improved
36	161298	43	F	Burns	40% burns	-	*E.coli*	R	R	R	R	R	R	S	R	R	R	+	-	+	+	+	ND	ND	Improved
37	157828	28	F	Burns	45% burns	-	*Proteus spp*	R	R	R	R	R	R	R	S	R	R	+	+	+	+	-	P	P	Improved

38	160968	60	M	Medicine	R- sided empyema	-	*Klebsiella spp.*	R	R	R	R	R	R	R	R	R	R	+	+	+	+	-	P	P	Improved
39	159119	51	M	Surgery	Cholecystectomy	-	*E.coli*	R	R	R	R	R	R	S	R	R	R	+	+	+	+	-	ND	ND	Improved
40	158742	44	F	Derm	Psoriasis vulgaris	-	*E.coli*	R	R	R	R	R	R	R	R	R	R	+	+	+	+	-	ND	ND	Improved

URINARY ISOLATES:

S No	IP no	Age	Sex	Ward	Diagnosis	Catherised / Not	Organism	G	AMP	COT	CIP	CTX	CFS	CX	AK	AT	NIT	MRP	KPC	MHT	EDS	CDT	AMPC	*bla* NDM-1	*bla* OXA-51	*Clinical condition*
1	193461	32	F	Ortho	ORIF	Catheterisd	*E.coli*	R	R	R	R	R	R	S	S	R	R	R	+	+	+	+	-	ND	ND	Improved
2	221220	56	F	Medicine	CRF	Uncatheterised	*E.coli*	R	R	R	R	R	R	S	R	R	R	R	+	+	+	+	-	ND	ND	Improved
3	214028	20	M	Surgery	Wound debridement	Catheterised	*Klebsiella spp.*	R	R	R	R	R	R	S	S	R	R	R	+	+	+	+	-	ND	ND	Improved
4	215254	64	F	ICU	CA palat	Catheterised	*Proteus spp*	R	R	R	R	R	R	R	R	R	R	R	+	-	+	+	+	ND	ND	Improved

					e																				
5	214211	65	M	ICU	MI infarcts	Catheterised	*Klebsiella spp*	R	R	R	R	R	R	S	R	R	R	R	+	+	-	-	+	ND	ND	Improved

SPUTUM

SNo	IP no	Age	Sex	Ward	Diagnosis	Comorbid condition	Organism	G	AMP	COT	CIP	CTX	CFS	CX	AK	AT	MRP	KPC	MHT	EDS	CDT	AMPC	*bla* NDM-1	*bla* OXA-51	*Clinical condition*
1	205371	85	M	CTB	Pneumonia	Post PT	*Klebsiella spp.*	R	R	R	R	R	R	R	R	R	R	-	-	-	-	+	N	N	Improved
2	204021	68	M	CTB	Ascites	-	*E.coli*	R	R	R	R	R	R	S	S	R	R	+	+	+	+	-	ND	ND	Improved
3	203156	68	M	Surgery	Multiple liver abscess	-	*E.coli*	R	R	R	R	R	R	S	R	R	R	+	+	+	+	-	ND	ND	Improved

4	207085	55	M	casualty	? PT	-	*Proteus spp*	R	R	R	R	R	R	S	R	R	R	+	+	+	+	-	ND	ND	Improved
5	204098	60	M	CTB	DCLD	PT	*Klebsiella spp.*	S	R	R	R	R	R	S	S	R	R	+	+	+	+	-	ND	ND	Improved
6	207767	55	M	Surgery	RIF mass/ COPD	-	*E.coli*	R	R	R	R	R	R	S	R	R	R	+	-	+	+	+	ND	ND	Improved
7	206221	74	F	Medicine	LRI/CAD	SHTN	*E.coli*	R	R	R	R	R	R	S	S	R	R	+	+	+	+	-	ND	ND	Improved
8	203364	55	F	Medicine	Bronchitis		*E.coli*	R	R	R	R	R	R	R	R	R	R	+	+	-	-	+	N	N	Improved
9	205381	60	M	CTB	LRI	Post PT	*Klebsiella spp.*	R	R	R	R	R	R	S	R	R	R	+	-	+	+	+	P	N	Improved
10	204240	68	M	CTB	?PT	-	*Proteus spp*	R	R	R	R	R	R	R	R	R	R	+	+	+	+	-	ND	ND	Improved
11	206221	74	F	Medicine	DCLD	-	*E.coli*	R	R	R	R	R	R	S	R	R	R	+	+	+	+	-	ND	ND	Improved

*-Only 25 isolates were subjected to genotyping. ND (Not done) indicates that the isolate has not been subjected to genotyping.

PROFORMA

CLINICAL DETAILS:

NAME: **AGE/SEX:**

S.No: **IP/OP NO:**

D.O.A:
ADDRESS: **D.O.D:**
OCCUPATION:

PRESENTING COMPLAINTS (WITH ANTIBIOTIC HISTORY):

PAST HISTORY:

Hospitalisation	**Treatment**	**Invasive procedure if any**

HISTORY OF ASSOCIATED ILLNESS: Diabetes/HIV/other co-morbid conditions

GENERAL PHYSICAL EXAMINATION:

PROVISIONAL DIAGNOSIS:

TREATMENT

INVESTIGATIONS DONE:
A) GENERAL:

B) **MICROBIOLOGY:**
Sample, date and site of collection:

Culture: **Gram Stain:**

Nutrient agar:

Blood agar:

Mac Conkey agar:

Chocolate agar:

GRAMS	
PIGMENT	
CATALASE	
OXIDASE	
MOTILITY	
INDOLE	
UREASE	
NR TEST	
MR TEST	
VP TEST	
Glucose	
Lactose	
Sucrose	
Maltose	
Mannose	
Xylose	
Lysine	
Arginine	
Orninthne	

Organism isolated:

ANTIBIOGRAM:

Antibiotic	Zone	Interpretation
G		
Amp		
COT		
CIP		
CTX		
CFS		
CX		
AK		
AT		
MRP		

Patient's response to treatment:

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